Effects of sterol composition on the lipids and properties of the plasma membrane of Saccharomyces cerevisiae.

Hossack, John Adrian

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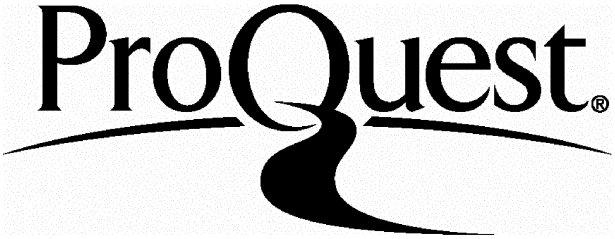
Effects of Sterol Composition on the Lipids and Properties of the Plasma Membrane of *Saccharomyces cerevisiae*

submitted by John Adrian Hossack
for the degree of Ph. D. of the
University of Bath

1975



SUMMARY

Saccharomyces cerevisiae NCYC 366, was grown anaerobically in batch culture, in the presence of one of a number of different sterols. Lipids were extracted from the cells and the sterol compositions analysed in order to find the degree of enrichment of the particular sterol provided in the medium. The free sterol content was found to range from 67% in the case of 7-dehydrocholesterol to 93% in cells grown in the presence of stigmasterol. The total lipid contents and the contents and compositions of other lipids were also analysed in order to find if any of the sterols in any way influenced lipid metabolism in the yeast. Lipids examined included free sterols, esterified sterols, phospholipids, triacylglycercls, squalene, and fatty-acyl compositions of phospholipids and neutral lipids. Variations among cells enriched with different sterols were only slight, the greatest range being in the sterol ester contents, where cells enriched in cholesterol had a content some three to ten times greater than other cells. An interesting result was that, in all cells, the fatty-acyl residues in the phospholipids consisted of 72 - 79% unsaturated residues, whereas there was less than 4% unsaturated residues in the neutral lipids. This finding is discussed in relation to the possible roles of lipid containing vesicles in phospholipid synthesis.

In order to check that the sterols were in fact being incorporated into the plasma membranes, these organelles were specifically labelled, isolated and their free sterol compositions measured. These showed that 70% of the

sterol present was chemically identical with that supplemented to the medium.

Sphaeroplasts of cells enriched in specific sterols were prepared, and their behaviour following suspension in hypotonic solutions of buffered sorbitol was measured. Two groups of responses were observed. Those sphaeroplasts enriched with a sterol possessing a double bond at C-22 remained relatively stable at 0.9 - 0.7M sorbitol before becoming susceptible to osmotic lysis, whereas those enriched with sterols without a double bond at C-22 lysed rapidly in sorbitol solutions below 1.0M.

The function of sterol structure and membrane stability, particularly with regard to stretching is discussed, and the data obtained from osmotic lysis compared to some preliminary data on phospholipid-sterol interactions as a monolayer at an air-water interface.

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INTRODUCTION

Yeasts, particularly strains of Saccharomyces cerevisiae, have for many years been popular organisms for studying biochemical phenomena. This is because they are eukaryotic, and yet, being unicellular, do not represent too specialized a system. They are easy to handle in the laboratory, and can be grown under a variety of environmental conditions. However knowledge of the lipids of yeasts is not so extensive as that of other cellular components, such as nucleic acids, proteins and carbohydrates. This is due, in part at least, to the physical properties of lipids which make them relatively difficult to work with. Recently, with the development of more accurate and sensitive techniques for lipid analysis, reliable data on yeast lipids have been reported.

Some fifty years ago the importance of lipids in membrane structure was suggested by Gorter and Grendel (1925). This discovery lead to further research on both the structure and composition of biological membranes. Progress has been greater on membrane composition rather than structure. However two main models of membrane structure have been proposed. Davson and Danielli (1935) suggested that the basic membrane consists of a bilayer, some 7.5 nm wide, which is seen in thin section in the electron microscope as an electron-transparent lipid layer sandwiched between two electron-dense layers of protein. Robertson (1959) dubbed this a "unit membrane". Although the unit membrane is an attractive model, other theories favour discontinuity within the plane of the membrane.

The amphipathic nature of the protein allows hydrophobic interaction with lipid fatty-acyl chains, while the polar groups protrude into the aqueous phase (Singer and Nicolson, 1972). This model is known as the fluid mosaic model.

The object of the present study was to examine aspects of the relationship between composition and function of yeast membranes, with particular reference to variation in the lipid composition. In any such study the ideal approach is to effect specific changes in the chemical composition of the organelle being studied. There are three ways in which specific changes in the lipid composition of membranes may be achieved. Firstly, the growth conditions may be altered in such a way that a single and hopefully stoicheiometric change is obtained. Secondly, mutants, deficient in an enzyme concerned with the biosynthesis of a particular membrane component, may be used and fed with the compound for which it is auxotrophic. Lastly, drugs which inhibit biosynthesis of a specific compound may be employed.

The main part of the research described in this thesis set out to examine how altering the sterol composition influences the lipid composition of Sacch. cerevisiae and also the stability of the sphaeroplasts formed from these cells.

In this work, use was made of the fact that Sacch. cerevisiae grown on a defined medium under anaerobic conditions

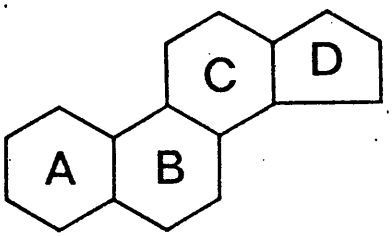
becomes auxotrophic for a sterol and an unsaturated fatty acid (Andreasen and Stier, 1954). Throughout this work Tween 80 was used as a source of oleic acid and by supplementing the medium with one of a variety of sterols, cells were obtained enriched in particular sterols.

YEAST LIPIDS

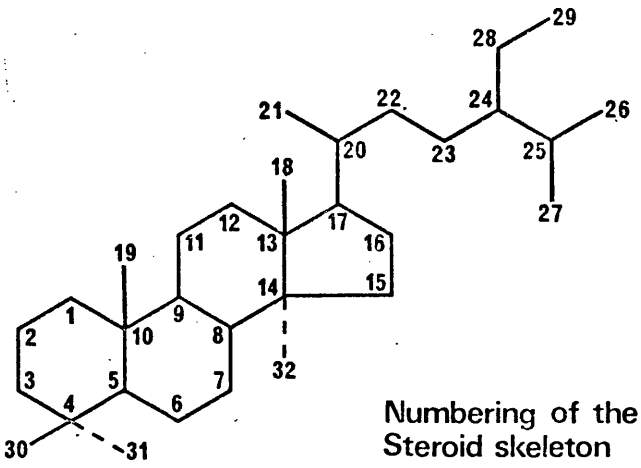
Considerable work has now been reported on the lipid composition of yeasts and, as a result, two main groups of yeast have been recognized. Most yeasts have a lipid content representing 7 - 15% of their dry weight, while a smaller group of these unicellular fungi contain much more lipid (30 - 60%). Lipids are difficult to define, but they are a heterogeneous group of compounds characterized by their variable solubility in organic solvents, and sparing solubility in water. Such properties reflect their hydrophobic nature. The lipids found in yeasts are those typically found in eukaryotic organisms. These include glycerophospholipids, sterols, sterol esters and triacylglycerols (Erwin, 1973).

Sterols

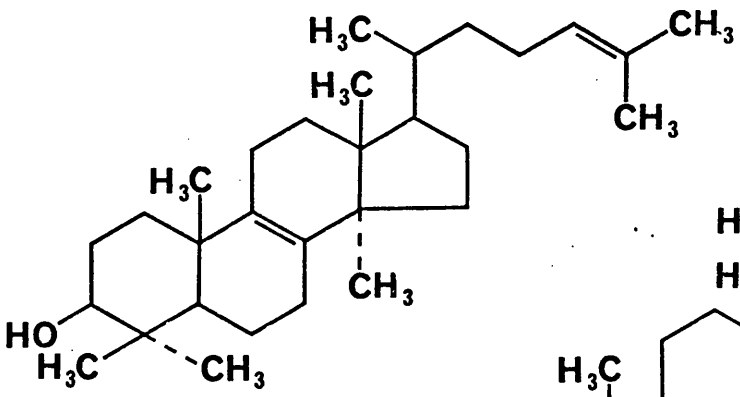
Sterols are complex molecules whose structures are based on the cyclopentanoperhydrophenanthrene ring (Fieser and



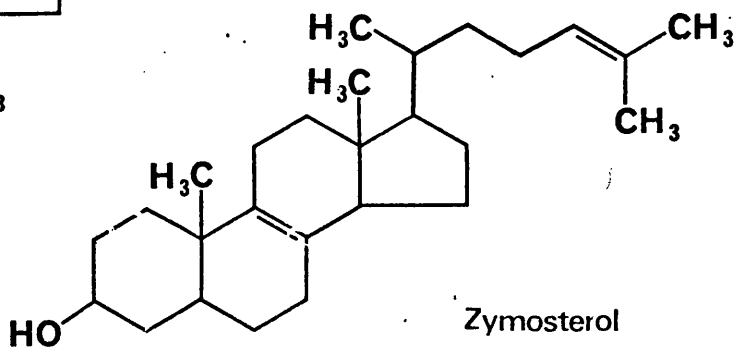
Cyclopentanoperhydro-phenanthrene



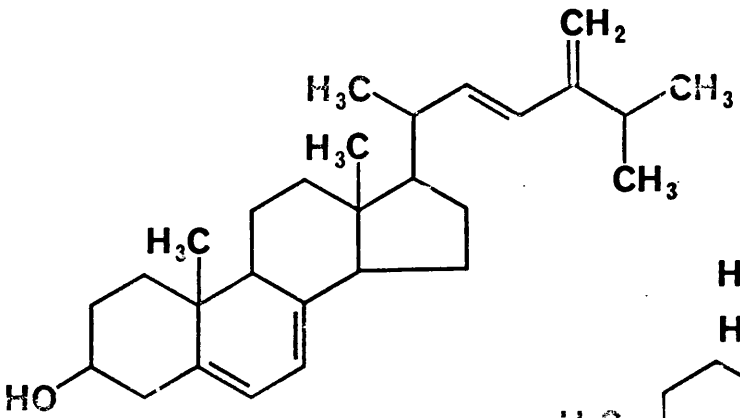
Numbering of the Steroid skeleton



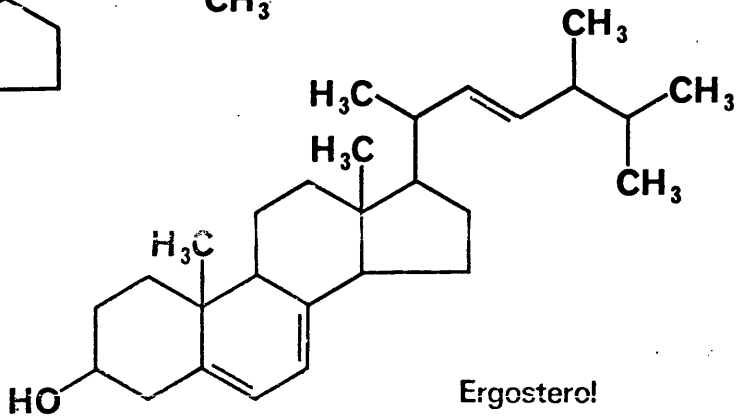
Lanosterol



Zymosterol



24(28)-Dehydro-ergosterol



Ergosterol

Fig.1. Formulae of some important yeast sterols

Fieser, 1959; Shoppee, 1964; Klyne, 1965).

The total sterol content of yeasts including the free alcohol and esterified forms usually comprises 0.1 to 2.0% of the dry weight (Bills et al., 1930; Shaw and Jefferies, 1953), although Dulaney et al. (1954) reported a strain of Sacch. cerevisiae which, under certain conditions, may contain 7 - 10% of its dry weight as sterols. The commonest yeast sterol is ergosterol (Hunter and Rose, 1971) which was first isolated from ergot-infected rye in 1889 (Tanret, 1889) and from yeast in 1895 (Gerard, 1895). Elucidation of its structure is reviewed by Fieser and Fieser (1959). Ergosterol is the principal sterol found in most yeasts which have been studied (Wieland and Benend, 1942; Usden and Burell, 1952; Dulaney et al., 1954). The next most common sterol found in yeast is 24 (28)-dehydroergosterol, first reported in baker's yeast by Breivik et al. (1954). This was found to be the major sterol in Sacch. cerevisiae N.C.Y.C. 366 by Longley et al. (1968), though Hunter and Rose (1972) using the same strain found ergosterol and 24 (28)-dehydroergosterol in about equal proportions. Zymosterol has also been reported in many yeasts (Wieland and Benend, 1942; Usden and Burell, 1952; Dulaney et al., 1954) and it has been suggested to be an intermediate in ergosterol biosynthesis (Fryberg et al., 1973). Other minor sterols reported in yeasts include lanosterol (Wieland et al., 1937), 7, 22 ergostadien-3β-ol, episterol, fecosterol (Wieland and Coutelle, 1941), mono- and dimethylzymosterol (Ponsinet and Ourisson, 1965), and

4α-methyl-8, 24 (28) ergostadien-3β-ol (Barton et al., 1968), some of which may be intermediates in ergosterol biosynthesis (Fryberg et al., 1973). Ascosterol (Wieland and Coutelle, 1941), cerevisterol and 14-dehydro-ergosterol (Fieser and Fieser, 1959) have also been detected in yeasts.

As well as being present as the free alcohols, sterols are also esterified with long chain fatty acids (Smedley-MacClean and Thomas, 1920; Maguigan and Walker, 1940). Madyastha and Parks (1969) analysed the fatty acids from the sterol esters of Sacch. cerevisiae, and found that C_{16} and C_{18} acids predominated with some C_{14} acids. An ergosterol-polysaccharide complex has also been reported to be present in baker's yeast. The binding between the sterol and the polysaccharide is non-covalent, and the polysaccharide is thought to be glycogen (Adams and Parks, 1968). Sterol glycosides, detected in cell envelopes of Sacch. cerevisiae, had a different sterol composition from other sterols, although a high proportion were unidentified (Työrinoja et al., 1974).

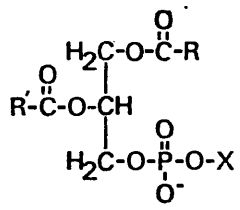
Phospholipids

Phospholipids are the substituted fatty-acyl diesters of sn-glycerol. The principal phospholipids in yeast are those typical of eukaryotes, namely phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidyl-

inositol, diphosphatidylglycerol (cardiolipin) and phosphatidylglycerol. Each represents a class of compounds which vary in the nature of the fatty-acyl residues on the glycerol moiety.

Numbering of the carbon atoms of glycerol is based on the stereochemistry of L-glyceraldehyde (Hirschmann, 1960). Phospholipids become 1,2-diacyl-sn-glycerophosphatides. Similarly triacylglycerols are designated triacyl-sn-glycerols (I.U.P.A.C. - I.U.B. Commission, 1963). The fatty acids found in phospholipids contain 8 - 24 carbon atoms, the major ones being C_{16} and C_{18}. These fatty-acyl residues may be saturated or unsaturated, and are normally straight-chain and even-numbered. Branched-chain residues and those containing cyclopropane rings, which are relatively common in prokaryotes, are absent or rare in yeast. Polyunsaturated fatty-acyl residues are usually present in small amounts in yeast, although $C_{18:2}$ and $C_{18:3}$ fatty-acyl residues represent 35% of those of Kluyveromyces (Sacch.) fragilis (Noble and Duitschaever, 1973). Long-chain fatty-acyl residues, with 20 to 30 carbon atoms, have also been reported in yeast; $C_{26:0}$ accounted for about 1% of the total fatty-acyl residues (Welch and Burlingame, 1973).

The major phospholipid of yeast is usually phosphatidyl-choline which represents 25 - 50% of the total phospho-lipids in Sacch. cerevisiae (Jollow et al., 1968; Longley et al., 1968; Getz et al., 1970; Hunter and



R,R′=Fatty-acyl residues

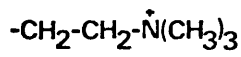
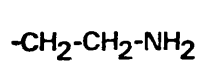
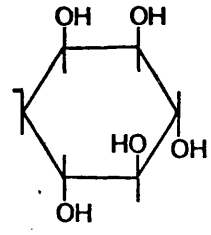
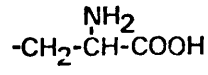
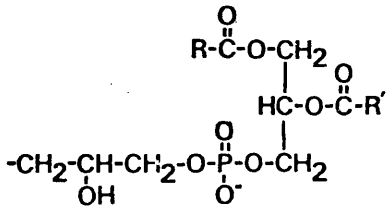
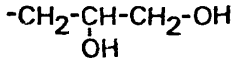
Structure of X	Name of Phospholipid
	Phosphatidylcholine
	Phosphatidylethanolamine
	Phosphatidyl<u>myo</u>-inositol
	Phosphatidylserine
	Diphosphatidylglycerol (Cardiolipin) R,R′=Fatty-acyl residues
	Phosphatidylglycerol

Fig.2. The structures of some glycerophospholipids found in yeasts

Rose, 1972). The next major yeast phospholipid is phosphatidylethanolamine, accounting for 20 - 32% of the total phospholipid in Sacch. cerevisiae (Longley et al., 1968; Getz et al., 1970; Hunter and Rose, 1972). Both of these phospholipids are prevalent in Sacch. carlsbergensis (Shafai and Lewin, 1968) and Candida sp. (Kates and Baxter, 1962). N,N-Dimethyl- and N-methyl-phosphatidylethanolamine have also been isolated from lipid extracts of Sacch. cerevisiae (Letters, 1966). These compounds are intermediates in synthesis of phosphatidylcholine from phosphatidylethanolamine (Steiner and Lester, 1970).

Phosphatidyl-myo-inositol was first isolated from yeast by Hanahan and Olley (1958), and accounts for about 20% of the phospholipids in Sacch. cerevisiae (Deierkauf and Booij, 1968; Longley et al., 1968; Getz et al., 1970; Hunter and Rose, 1972) and Sacch. carlsbergensis (Shafai and Lewin, 1968). Mono- and di-phosphoinositols have also been detected in Sacch. cerevisiae (Lester and Steiner, 1968), and these phospholipids are believed to be the equivalent of the 4-phospho and 4,5-diphosphomono-esters found in rat brain. Further evidence for the presence of such compounds in yeast is the finding of phosphatidylinositol kinase activity in Sacch. cerevisiae (Wheeler et al., 1972). These esters are, however, present in much smaller amounts than phosphatidylinositol (Steiner and Lester, 1972).

Compared with these phospholipids, phosphatidylserine is usually present in smaller proportions in Sacch. cerevisiae (Deierkauf and Booij, 1968; Getz et al., 1970; Hunter and Rose, 1972), where it represents about 4% of the total phospholipids. However, in Lipomyces starkeyi, the value may be as high as 18% (Hunter and Rose, 1971).

Diphosphatidylglycerol (cardiolipin) was identified in Sacch. cerevisiae by Letters (1966), and usually accounts for about 4% of the phospholipids of this species (Deierkauf and Booij, 1968; Getz et al., 1970; Hunter and Rose, 1972). The same lipid is also present in Candida sp. (Kates and Baxter, 1962). Cardiolipin has been found to be located mainly in mitochondria in Sacch. cerevisiae (Jakovcic et al., 1971). Another glycerophospholipid found in about the same proportion as cardiolipin is phosphatidylglycerol, which was first isolated from Sacch. cerevisiae by Letters (1966). Phosphatidylglycerol phosphate has been found in lipid extracts from mutant strains of Sacch. cerevisiae by Deierkauf and Booij (1968) and is believed to be an intermediate in the biosynthesis of cardiolipin.

Lysophospholipids are phospholipids which do not have a fatty-acyl residue on C-1 or C-2 of sn-glycerol (Tattrie and Cyr, 1963). Many of the lysophospholipids encountered in yeast may be artefacts, due to the action of phospholipases during extraction, particularly in anaerobically-

grown cells (Letters, 1968a). Letters found that lipids of Sacch. cerevisiae contained a high proportion of lysophosphatidylcholine and lysophosphatidylethanolamine when the cells were grown anaerobically but not when cultured under aerobic conditions. Getz et al. (1970) found only 1% of lysophospholipids in Sacch. cerevisiae grown aerobically.

Another phospholipid found in extracts of yeast which could arise as a result of phospholipase D activity is phosphatidic acid. This compound has been found in Sacch. cerevisiae (Longley et al., 1968) and Sacch. carlsbergensis (Shafai and Lewin, 1968) but not in Candida lipolytica (Kates and Baxter, 1962).

Amino-acyl esters of phosphatidylglycerol, usually referred to as lipo-amino acids, have been detected in bacteria, the first to be detected being lysylphosphatidylglycerol (MacFarlane, 1964). However, such lipids have not been found in yeasts or other eukaryotic organisms.

Triacylglycerols

These are esters of the trihydric alcohol, sn-glycerol and long-chain fatty acids; the fatty acids are the same as those found esterified in phospholipids. It is usual to find that the fatty-acyl residue at C-2 of sn-glycerol is

unsaturated, as in mammalian lipids (Meyer and Bloch, 1963a). Lipid particles have been isolated from Sacch. cerevisiae of which 90% of the lipid was triacylglycerols together with sterol esters (Clausen et al., 1974). Since they had a high proportion of unsaturated fatty-acyl residues they suggested these particles were a reserve of materials for membrane biosynthesis.

Diacyl-sn-glycerols (both 1,2 and 1,3) and monoacyl-sn-glycerols have also been found in yeast lipids (Kates and Baxter, 1962). They are possibly degradation products of triacyl-sn-glycerols as a result of lipase activity (Nurminen and Suomalainen, 1970) or of phospholipids by phospholipase C (Harrison and Trevelyan, 1963).

Hydrocarbons

These are probably the least understood of the yeast lipids. They can represent 2 - 20% of the total lipids of yeast (Kováč et al., 1967; Baraud et al., 1967). However accurate data have not been obtained, due to lack of reliable methods of determination. Baraud et al. (1967), using gas-liquid chromatography, separated hydrocarbons from Sacch. oviformis, either grown aerobically or anaerobically, and from Sacch. ludwigii grown anaerobically, and they found several different chain lengths, ranging from 10 to 31 carbon atoms. Branched hydrocarbons were also detected. Squalene, the C_{30} hydrocarbon precursor of

sterols, has been found in Sacch. cerevisiae (Jollow et al., 1968) and Sacch. carlsbergensis (Shafai and Lewin, 1968). In aerobically-grown cells it accounted for 2% of the total lipid, but this value was much higher in anaerobically-grown cells, even if the medium was supplemented with sterol. This is presumably due to an accumulation of squalene, which would normally require the presence of molecular oxygen before its cyclization to yield sterol.

Sphingolipids

These are the hydroxy-fatty-acyl esters of long-chain amino alcohols, and are found in small amounts in many yeasts. In yeasts, the type of sphingolipid found is referred to as a cerebroside (Hunter and Rose, 1971), which are amides of long-chain amino alcohols, substituted with sugars.

Cerebrosides were first reported in Sacch. cerevisiae by Reindel et al. (1940). Those from Torulopsis utilis were examined by Stanacev and Kates (1963) and, on hydrolysis, yielded C_{18} and C_{20} phytosphingosines and a small amount of C_{18} dihydrosphingosine. The main fatty acids were C_{18}, C_{24}, and C_{26} α-hydroxy-acids. Weinert et al. (1973) isolated, from Sacch. cerevisiae, three types of cerebroside which contained either a C_{18} sphingosine, a C_{18} or C_{20} dihydrosphingosine, or a C_{18}, C_{19}, or C_{20} phytosphingosine. The fatty-acyl residues found were

$C_{26:0}$, 2α-hydroxy-$C_{26:0}$ and 2,3-dihydroxy-$C_{26:0}$ acids.

Other workers have reported the presence, in baker's yeast, of sphingolipids containing inositol and mannose (Trevelyan, 1968; Nurminen and Suomalainen, 1971), and with C_{26} and 2-hydroxy-C_{26} fatty-acyl residues (Nurminen and Suomalainen, 1971). Steiner et al. (1969) extracted with pyridine a complex sphingolipid from Sacch. cerevisiae. Partial analysis revealed the compound to be mannosyl-di-inositol-diphosphatidylceramide. Acid or base hydrolysis released an inositol-phosphate moiety, and left a structure similar to the 'mycoglycolipid' found by Wagner and Zofcsik (1966) in Sacch. cerevisiae and Candida utilis. More recently a mono-inositol phosphorylceramide has been isolated from Sacch. cerevisiae by Smith and Lester (1974).

Polyprenols

These are polymers of isoprenoid units, $[-CH_2-C(CH_3)=CH-CH_2-]_n$, with a free hydroxyl group at one terminal, where n is between 5 and 24. The principal types found in yeast are the dolichols, which have a saturated unit at the hydroxyl end of the molecule (Hemming, 1970). Dunphy et al. (1967) isolated polyprenols from yeast, and found five main types containing 14 to 18 isoprenoid units, three of which are believed to be the trans configuration, while the remainder are cis.

Lipophilic intermediates are thought to be involved in the synthesis of mannan in the cell wall of Sacch. cerevisiae (Sentandreu and Elorza, 1973). The lipid acceptor was shown to be dolichol monophosphate in yeast (Tanner et al., 1971). As well as mannose, glucose and galactose have also been shown to form a dolichol-sugar complex in Sacch. cerevisiae (Palamarczyk and Chojnacki, 1973) which suggests a general role of polyprenolphosphate-sugars in yeast, rather than a specific transfer of mannose residues.

Glycolipids

Mono-galactosyl-diacylglycerol has been detected in Sacch. cerevisiae by Baraud et al. (1970). This is similar to the glycosyl di-acyl glycerol glycolipids found in bacteria by Shaw and Baddiley (1968).

EFFECTS OF GROWTH CONDITIONS ON THE COMPOSITION OF YEAST LIPIDS

The lipid composition of yeast is known to vary with changes in environmental conditions. These conditions include age of the culture, growth rate, growth temperature, medium composition and oxygen tension of the medium.

Age of the Culture and Growth Rate

Typically the lipid content of yeasts increases with age of the cultures. This has been found in Candida utilis (Dawson and Craig, 1966) and Sacch. cerevisiae (Castelli et al., 1969a). Of the individual lipids, the total phospholipids were found to be greatest during the exponential phase of growth in Sacch. cerevisiae (Castelli et al., 1969a; Getz et al., 1970). Dawson and Craig (1966) examined the fatty-acyl residues and lipids of Candida utilis and found an increased proportion of $C_{18:2}$ and $C_{18:3}$ acids as exponential growth progressed. They also observed a greater proportion of these acids in Candida utilis grown at a slower growth rate in a chemostat.

Growth Temperature

In considering the influence of growth temperature it must be remembered that a change in temperature also changes the rate of growth and the content of dissolved oxygen, unless the microbe is grown in a chemostat with control of oxygen tension.

Lowering of growth temperature causes an increase in the lipid content of Candida lipolytica (Kates and Baxter, 1962) and Sacch. cerevisiae, harvested at the mid exponential phase of growth (Hunter and Rose, 1972). Lipids of

Candida sp. grown at suboptimum temperatures contain greater proportions of unsaturated fatty-acyl residues (Kates and Baxter, 1962). This effect was also found in Sacch. cerevisiae by Chang and Matson (1972). These workers reported a greater proportion of fatty-acyl residues with chain lengths shorter than 16 carbon atoms at high temperatures. In contrast Hunter and Rose (1972) observed little difference in the composition of fatty-acyl residues of Sacch. cerevisiae grown at 30°C or 15°C, at constant growth rate.

The total phospholipid content has been found to be greater at lower growth temperatures in Candida lipolytica (Kates and Baxter, 1962) and Sacch. cerevisiae (Hunter and Rose, 1972). The latter workers also found that, in Sacch. cerevisiae grown at a fixed rate, a drop in temperature caused a large diminution in the total free and esterified sterol. Starr and Parks (1962a) demonstrated that, in Sacch. cerevisiae, the rate of sterol biosynthesis was optimum at 30°C, which is the optimal growth temperature. Above 40°C sterol synthesis was inhibited, but addition of sterol to the culture medium would support growth.

Medium Composition

The composition of the medium is also important in determining the lipid composition of yeast. The total lipid and

total fatty-acyl residues increased in Candida utilis, grown in a chemostat, if the glucose content of the medium was increased from 0.1 to 4.5% w/v (Babij et al., 1969). Conversely Brown and Johnson (1970) found that the total lipids and unsaturated fatty-acyl residues of Sacch. cerevisiae fell when the glucose content was increased. Mangnall and Getz (1973) observed that cardiolipin production was sensitive to the carbon source used in the medium. If Sacch. cerevisiae was transferred from a glucose-supplemented to a galactose-supplemented medium, the cardiolipin content increased, as did respiratory capacity. However Jakovcic et al. (1971) found similar changes in a respiratory deficient mutant of Sacch. cerevisiae, indicating that loss of respiratory activity does not bear a simple relationship to cardiolipin content.

Vitamin deficiency also affects the lipid composition of yeast. Haskell and Snell (1965) found less total lipid and a lower proportion of unsaturated fatty-acyl residues in pyridoxine-deficient Hanseniaspora valbyensis than in cells grown on medium optimally supplemented with the vitamin. Similarly pantothenate-deficient Sacch. cerevisiae has a lower total lipid content than cells grown in the presence of an optimal concentration of this vitamin (Furukawa and Kimura, 1971; Hosono and Aida, 1974). Inositol-deficient Sacch. carlsbergensis synthesizes a greater amount of triacylglycerols, but less phosphatidylinositol than in organisms grown in the

presence of optimal concentrations of inositol (Shafai and Lewin, 1968). Biotin deficiency in aerobically-grown Sacch. cerevisiae restricts synthesis of C_{18} fatty acids, particularly $C_{18:1}$ acids (Lynen, 1967).

Phosphate limitation of growth of Sacch. cerevisiae caused an increase in the content of total lipid and fatty-acyl residues, principally in the triacylglycerols, compared with carbon-limited cells (Johnson et al., 1973). However little change occurred in the composition of the fatty-acyl residues. Similar results were obtained with Candida utilis, but this organism showed a difference in the composition of fatty-acyl residues. Saccharomyces cerevisiae grown on a medium containing 0.1% yeast extract, and 1.0% glucose produced less sterol than if grown on medium containing 1.0% yeast extract and 2.0% w/v glucose (Starr and Parks, 1962b).

Oxygen Tension

In this thesis, the influence of anaerobiosis was exploited in order to obtain specific changes in the lipids of Sacch. cerevisiae. Yeast, grown anaerobically, becomes auxotrophic for a sterol and an unsaturated fatty acid (Andreasen and Stier, 1954), since the biosynthesis of these compounds requires the presence of molecular oxygen. The oxygen-requiring reactions in the biosynthetic pathways of these compounds are, respectively, those by which

squalene is cyclized, and in which a double bond is introduced into a saturated fatty-acyl residue.

Conversion of Squalene to Sterol Cyclization of squalene involves two different enzymes. The first stage is oxidative and requires $NADPH_2$, the product being squalene 2-3 oxide. The oxide is cyclized anaerobically to form lanosterol, catalysed by 2-3 oxido-squalene lanosterol cyclase. These enzymes have been isolated from rat liver preparations (Willet et al., 1967) and the cyclase was most active in the microsomal fraction. The cyclase activity has since been found in Sacch. cerevisiae (Barton et al., 1968).

Desaturation of Fatty Acids Bloch and his colleagues have isolated from Sacch. cerevisiae (Bloomfield and Bloch, 1960) and Candida utilis (Meyer and Bloch, 1963b) a microsomal enzyme system which desaturates coenzyme A esters of $C_{16:0}$ and $C_{18:0}$ fatty acids to the $C_{16:1}$ and $C_{18:1}$ derivatives, the reactions requiring molecular oxygen (Bloomfield and Bloch, 1960). Lennarz and Bloch (1960) suggested that a hydroxy acid may be an intermediate in this oxygenase-catalysed reaction, though such compounds were not converted to oleic acid (Light et al., 1962). The purified desaturase from baker's yeast, free of synthetase activity, desaturated palmitoyl-CoA without prior transfer of the fatty-acyl residue to the enzyme, suggesting a lack of involvment of an acyl-carrier protein. Since the desaturase also catalysed desaturation of the

coenzyme-A ester of $C_{10:0}$ acids, the production of mainly $C_{16:1}$ and $C_{18:1}$ acids in yeasts could be due, not to specificity of the desaturase, but to that of the synthetase which supplies coenzyme-A esters of $C_{16:0}$ and $C_{18:0}$ acids as substrates (Schultz and Lynen, 1971).

Serial desaturation has been shown to give rise to polyunsaturated acids such as $C_{18:2}$ in Candida utilis (Yuan and Bloch, 1961) from which the enzyme responsible was isolated (Meyer and Bloch, 1963b). It is specific for coenzyme-A esters of $C_{18:1}$ acids, and requires molecular oxygen and $NADH_2$ or $NADPH_2$ as cofactors, as well as an unidentified factor, not required for the formation of mono-unsaturated acids (Meyer and Bloch, 1963b).

However it is worth noting that microsomal fractions of Candida lipolytica (Pugh and Kates, 1973) and Torulopsis utilis (Talamo et al., 1973) have been shown to catalyse desaturation of fatty-acyl residues on phosphatidylcholine or phosphatidylethanolamine, in the presence of molecular oxygen and $NADPH_2$ or $NADH_2$, but not requiring CoA,SH or ATP, as does the synthetase.

Several groups of workers, notably that led by Linnane, have reported on the lipid compositions of anaerobically-grown Sacch. cerevisiae. Sacch. cerevisiae, grown anaerobically in a medium supplemented with a sterol and unsaturated fatty acid, had a lower total lipid than

aerobically grown cells (Jollow et al., 1968). A similar result was found with a respiratory-deficient mutant of Sacch. cerevisiae (Kovác et al., 1967). Individual lipids also vary in response to anaerobiosis or lowered oxygen tension in the medium. Anaerobically-grown Sacch. cerevisiae was found to have less total fatty-acyl residues, total phospholipids and sterol contents (Jollow et al., 1968). However, the squalene content was greater in anaerobically-grown cells; this is presumably due to the requirement of molecular oxygen for its cyclization during sterol biosynthesis.

The fatty-acyl composition is known to differ dramatically between aerobically and anaerobically-grown cells. Anaerobically-grown cells contain a high proportion of short-chain saturated acids, containing 10 to 14 carbon atoms, rather than longer-chain unsaturated acids (Jollow et al., 1968).

The fatty-acyl residue composition of yeast also varies under conditions of lowered oxygen tension. Candida utilis, grown in a chemostat under glucose limitation, had less saturation in the fatty-acyl residues, but more short-chain acids, when the oxygen tension was lowered from 75 to <1 mm mercury, the greatest change occurring at tensions of 1 mm of mercury and lower (Brown and Rose, 1969). Chang and Matson (1972) found a much higher proportion of fatty-acyl residues with 10 to 14 carbon atoms in semi-anaerobically grown Sacch. cerevisiae than

in aerobically-grown cells. Mono-unsaturated acids ($C_{16:1}$ and $C_{18:1}$) represented about 45% of the total fatty-acyl residues of aerobically-grown cells, but were present in only trace amounts in semi-anaerobically-grown cells.

Of the individual phospholipids, phosphatidylethanolamine and cardiolipin are found to be in much lower amounts in anaerobically-grown Sacch. cerevisiae (Jollow et al., 1968; Getz et al., 1970). Jollow et al. (1968) reported greater proportions of phosphatidylcholine and phosphatidylinositol in anaerobically-grown cells. There is a higher proportion of lysophospholipids in anaerobically-grown Sacch. carlsbergensis (Letters, 1968a), but this was much lower in Sacch. cerevisiae (Getz et al., 1970).

The sterol content of anaerobically-grown Sacch. cerevisiae is greatly lowered and, under strict anaerobic conditions, the medium must be supplemented with a sterol (Andreasen and Stier, 1954). In Sacch. cerevisiae grown anaerobically in sterol-supplemented medium, Jollow et al. (1968) reported only 0.07 mg sterol per 100 mg dry weight cells, whereas aerobically grown cells contained 0.82 mg per 100 mg dry weight of cells. The yeast Schizosaccharomyces japonicus can grow anaerobically without sterol supplement and the sterol content is then 0.01 mg per 100 mg dry weight cells, compared with a value of 0.24 mg per 100 mg dry weight cells for aerobically-grown cultures (Bulder, 1971).

Adams and Parks (1967) found that sterols of Sacch. cerevisiae appeared to be of two types; one was acid-labile, the other base-labile. They noticed that the acid-labile type increased and the base-labile sterol decreased at early adaptation to aerobic respiration, and suggested that the proportions of each would depend on the respiratory state of the cells.

STRUCTURE AND FUNCTIONS OF MEMBRANES

A membrane is a pliable, selectively permeable, barrier, surrounding a cell or cell organelle. Prokaryotic organisms have a relatively simple membrane system, consisting of only a plasma membrane, whereas eukaryotes, including yeasts, possess a complex system of intracellular membranes associated with organelles, as well as the plasma membrane. The principal functions of membranes are considered to be threefold: firstly, to act as a barrier between the internal environment of a cell or organelle and its external environment, and so to give it an organised structure; secondly, to regulate transport of compounds into or out of the cell or organelle; and thirdly to be involved in or to provide a site for certain enzymatic processes. As long ago as 1899, Overton, from permeability studies on plant tissues, postulated that membranes contained a fatty substance (Overton, 1899). Analyses of membranes have subsequently shown that they are composed mainly of lipid and protein.

The major membrane lipids are phospholipids and sterols. In prokaryotic organisms, sterols are absent, and the phospholipids are mainly phosphatidylethanolamine and phosphatidylglycerol as diphosphatidylglycerol (Erwin, 1973). Eukaryotes, including yeasts, also possess phosphatidylcholine, phosphatidylserine and phosphatidyl-inositol. There is little sphingolipid in yeast, though these lipids may be important in mammalian membranes. Sterols vary in structure in different organisms. In animals, cholesterol is the predominant one; higher plants contain mainly stigmasterol and β-sitosterol whereas ergosterol is the most common sterol in yeasts (Weete, 1973).

The lipid composition of yeast membranes also varies, presumably associated with specificity of function. For example, most of the cardiolipin in Sacch. cerevisiae is present in mitochondrial membranes (Jakovcic et al., 1971). However few data are available for the exact lipid composition of yeast plasma membranes, mainly due to difficulties in obtaining pure preparations.

Isolation of Yeast Plasma Membranes

The existence of a system of intracellular membranes in yeast has been demonstrated by electron microscopy (Matile et al., 1967), but no completely satisfactory isolation of plasma membrane has yet been achieved from yeast.

There are two main methods which can be used to isolate plasma membrane from yeast. Boulton (1965) removed the cell wall of Sacch. cerevisiae with the digestive juice of Helix pomatia (Eddy and Williamson, 1957). The sphaeroplasts so formed were subjected to osmotic lysis for 30 min at 0°C in 0.025M tris buffer (pH 7.2) containing mM $MgCl_2$. The plasma membrane and cell debris were obtained by centrifugation at 20,000g for 30 min. Of the membrane fraction, 90% of the dry weight was lipid plus protein. Osmotic lysis was also used on sphaeroplasts of Candida utilis by Garcia-Mendoza and Villaneuva (1967). They found that analyses of the membranes yielded mainly protein and lipid. A more detailed lipid analysis of the membrane fraction of Sacch. cerevisiae, obtained by the same method, was carried out by Longley et al. (1968). They found that protein and lipid accounted for 50% and 40%, respectviely, of the dry weight. The remainder comprised nucleic acids and carbohydrates. The lipids were those typical of membranes, namely phospholipids and sterols.

Other workers claim that membranes prepared by osmotic lysis of sphaeroplasts are heterogeneous, and that the analyses carried out do not give representative results. Matile et al. (1967) homogenized Sacch. cerevisiae in 0.05M tris buffer containing 0.25M sucrose and mM EDTA. The cell-free extract was submitted to differential centrifugation, and the microsomal fraction, which separated from the mitochondrial fraction at 150,000g for

30 min, was layered on a density gradient of Urografin. The plasma membrane was separated as a distinct band at 1.165 - 1.170 g/cm^3. Analyses again identified lipid and protein, although quite considerable amounts of carbohydrate were present.

Suomalainen and his coworkers have isolated plasma membrane by digestion with snail juice, of isolated cell envelopes, which consist of cell wall with attached plasma membrane, obtained by breaking baker's yeast in a disintegrator (Nurminen et al., 1970). They have reported on the lipid composition of these preparations (Suomalainen and Nurminen, 1970).

The digestive juice of Helix pomatia (which is also known as helicase) is a mixture of some 30 enzymes (Holden et al., 1950), amongst which is known to be lipase, so that caution must be employed when it is used. To overcome this objection, Nurminen et al. (1970) and Suomalainen and Nurminen (1970) removed lipase and phospholipase by subjecting the juice to gel filtration on Sephadex G.100 in 0.85% (w/v) NaCl.

Problems with obtaining pure preparations of plasma membranes are those of fragmentation (Boulton, 1965) and vesiculation (Dubé et al., 1973). Recently Shibeci et al. (1973) prepared sphaeroplasts of Sacch. cerevisiae with helicase, and labelled the outer membrane with various radioactively labelled reagents, selected for various amino-acid residues

of the membrane proteins. The labelled sphaeroplasts were recovered by centrifugation, washed in mannitol buffer, and membranes obtained by lysis in 0.1M phosphate buffer (pH 7.0) containing 10mM $MgCl_2$. The membrane preparation was layered onto a discontinuous sucrose-density gradient and separated at 189,000g for 2.5 hours at 4°C. The bulk of the radioactivity was found in bands with a density of 1.18 - 1.29g/cm^2.

Methods for Effecting Changes in the Lipid Composition of Yeast Membranes

As already mentioned, to examine the structural requirements for the incorporation of lipids in membranes, one of the following methods should be used to effect specific alterations in membrane composition. (a) Variation of the environmental conditions, so that a specific pathway does not function and therefore a specific lipid component is not synthesised. This lipid can then be supplied in the medium; (b) Use of mutants deficient in the ability to synthesise certain lipids, and which are therefore auxotrophic for these lipids; (c) Use of drugs as specific inhibitors of a pathway leading to synthesis of a lipid. The extent to which method (c) has been used is very slight and will not be referred to any further.

As far as mutants are concerned, most of the work has been carried out with mutants which are auxotrophic for a fatty acid. There are two classes of fatty acid-requiring

mutants of Sacch. cerevisiae. Firstly there are the desaturase mutants, which lack the capacity for desaturating saturated acids ($C_{16:0}$ and $C_{18:0}$) at the C_9 - C_{10} bond. Secondly there are the chain-lengthening mutants, which cannot elongate the fatty-acyl chain. The earliest mutants to be isolated in the first class were by Resnick and Mortimer (1966). The specificity of chain length for such mutants was found to be broad (Wisnieski and Kiyomoto, 1972), though rather less broad with cis or trans acids. Williams et al. (1973), using a desaturase mutant of Sacch. cerevisiae, supplemented various polyunsaturated C_{20} acids and found growth was best if the acid contained double bonds at C_5, C_8 and C_{11}. With regard to the second group of fatty acid-requiring mutants, recent work has been concerned with discovering the range of saturated fatty acids which can be utilized (Henry, 1973).

Sterol-requiring mutants of yeasts have proved much more difficult to obtain, though recently sterol-requiring respiratory-deficient mutants of Sacch. cerevisiae have been isolated (Karst and Lacroute, 1973; Gollub et al., 1974). The former workers have also isolated a sterol-requiring mutant which is not petite and which possibly has a block at the enzymatic stage converting squalene to lanosterol (Karst and Lacroute, 1974). The requirement is not stringent for ergosterol as growth will occur with a supplement of cholesterol, β-sitosterol or stigmasterol (Karst and Lacroute, 1973).

With reference to the first method for effecting specific changes in yeast lipids, when Sacch. cerevisiae is grown anaerobically it becomes auxotrophic for an unsaturated fatty acid and a sterol (Andreasen and Stier, 1954). A low order of specificity was found for C_{16} and C_{18} acids when provided for anaerobically-grown Sacch. cerevisiae (Light et al., 1962), since acids with a hydroxyl group, a cis double bond, a triple bond, or a cyclopropane group in the central region of the molecule each supported growth. Proudlock et al. (1968) also found a low order of specificity for sterol structural requirements, in order to support anaerobic growth of Sacch. cerevisiae. They found that the three structural requirements of the steroid molecule were a planar steroid nucleus, a hydroxy group at C-3, and a long alkyl side chain at C-17. Interestingly, this is in contrast to the structural requirements of sterols for T-strain Mycoplasma (Rottem et al., 1971), where a sterol with a saturated, rather than an unsaturated, side chain was necessary for satisfactory growth.

Phospholipid-Sterol Interactions

The precise arrangement of phospholipids and sterols in membranes is not fully understood, and several physical techniques have been employed to investigate this arrangement, and after to explain the exact function of the sterol component. Work has been carried out on

artificially prepared monolayers and bilayers, as well as on biological membranes.

The fatty-acyl chains in phospholipids exist in two forms, namely the crystalline and liquid-crystalline phases. Conversion of the crystalline to the liquid-crystalline form (melting), as the temperature increases, in fact occurs below the capillary melting point of a hydrocarbon of comparable structure. This phenomenon is responsible for the fluidity of the membrane, and could be important in such membrane properties as permeability.

Nuclear magnetic resonance (n.m.r.) has been used to investigate the mobilities of fatty-acyl chains in phospholipids, phospholipid-sterol mixtures, and in mixtures of lipids from membrane. Oldfield et al., (1971), studying lecithin in water, found that the $\overset{+}{N}(CH_3)_3$ region of lecithin was highly mobile. However the fatty-acyl residues showed restricted mobility of methylene groups near the carboxyl end and greater freedom of movement at the methyl-group end of the residue (Chan et al., 1971).

Oldfield and Chapman (1972a) carried out n.m.r. studies on a mixture of sphingomyelin and 50 mole% cholesterol, and observed that, above the phase transition temperature of the fatty-acyl residue, cholesterol restricted motion, indicating a 'rigidizing' effect; but below the transition temperature, cholesterol 'fluidized' the chain.

The mobility of the 'intermediate fluid' state was insensitive to temperature between 20°C and 60°C. Similar results were also obtained with sonicated mixtures of lecithin dispersions and 50 mole % cholesterol over the same temperature range (Darke et al., 1971). They suggested that, in fact, lecithin and cholesterol formed a complex bound by van der Waals forces between the steroid nucleus and the first 10 methylene groups in each lecithin fatty-acyl residue, but leaving the remainder of the chain free. There is also hydrogen bonding between the 3β hydroxyl group of the sterol and the phosphate of the lecithin. These workers suggested that the complexes are present in clusters, rather than distributed evenly throughout the membrane.

The results of n.m.r. studies have been supported by electron-spin resonance (e.s.r.) data. These have been carried out on bilayers from liposome dispersions (Hubbell and McConnell, 1971; Oldfield and Chapman, 1971) and oriented systems (Jost et al., 1971). Motion in the bilayers is greatest towards the centre of the bilayer. Jost et al. (1971) also found that motion was greater at the methyl-group end rather than the carboxyl end of the fatty-acyl residues. This finding is consistent with data from e.s.r. on plasma-membrane lipids of Mycoplasma laidlawii (Rottem et al., 1970). Hydration of lecithin increased the chain motion, but this is most apparent at the carboxyl end of the molecule (Jost et al., 1971). Boggs and Hsia (1972) examined the influence of

hydration on the mobilities of fatty-acyl residues in dipalmitoyl lecithin and egg lecithin, and found that hydration increased the mobilities of each. This effect is achieved by decreasing the 'degree of order' of the phospholipid, i.e. the extent to which the fatty-acyl residues orientate themselves to the plane of the membrane. Rottem *et al.* (1970), using plasma-membrane lipids from *Mycoplasma laidlawii*, found that *cis* $C_{18:1}$ acids had more motion than the corresponding *trans* acids.

The effect of addition of sterols to lipids has also been examined by e.s.r. Hubbell and McConnell (1971), who used egg lecithin plus 33 mole % cholesterol, considered that the first eight carbon atoms from the carboxyl end of the fatty-acyl residue of the phospholipids exist as a rigid rod in the presence of cholesterol, with increasing motion towards the methyl-group end of the chain.

As with n.m.r., the 'rigidizing' and 'fluidizing' roles of cholesterol have been observed with e.s.r. At 20°C cholesterol made dipalmitoyl lecithin more fluid, presumably by relaxing the close packing of the saturated chains, but egg lecithin, which has a lower phase transition temperature, is made less mobile (Oldfield and Chapman, 1971; Schreirer-Mucillo *et al.*, 1972). Each mixture has a similar 'intermediate fluidity'. Above the transition temperature, a concentration of 50 mole % cholesterol was found to lower the mobility of fatty-acyl chains in egg-yolk lecithin, but the same effect was

obtained with only 25 mole % with dipalmitoyl lecithin (Boggs and Hsia, 1972). Above 25 mole % cholesterol, the sterol perturbed the close packing of the saturated chains. The deviation of the perpendicularly orientated phospholipid has been measured. Lapper et al. (1972) calculated, for egg-yolk lecithin, movement through 46° in the absence of cholesterol, decreased to a minimum of 17° in the presence of 55 mole % of the sterol.

The features of the sterol molecule required to cause maximum 'degree of ordering' of phospholipids have been examined by e.s.r. Hsia et al. (1972) found that,, with egg-yolk lecithin, the most effective sterols were cholesterol, 7-dehydrocholesterol, and lathosterol. Ergosterol and β-sitosterol were less effective. No effect was observed if the hydroxyl group was esterified, or replaced by a keto group, or if the alkyl side chain at C-17 was absent. Butler et al. (1970) found, with sterol-free brain lipids, that cholesterol had a greater effect than ergosterol at 25 mole %, but vice versa at 10 mole %. Above this latter value the bulkier side chain of ergosterol caused increased mobility. β-Sitosterol had a similar effect to cholesterol. These workers also found that replacing the 3β-hydroxyl group of the sterol, even by a 3α-hydroxyl, did not give an 'ordering' effect. If the side chain was absent at C-17 then the ordering was very much diminished. Further they found that there was no necessity for a double bond at C-5 but that, if the A-B ring fusion was trans

resulting in a non-planar nucleus, the ordering was very much decreased. Both groups of workers stated that, for maximum ordering of a phospholipid, a sterol molecule must have a 3β-hydroxyl group, a planar steroid nucleus, and a hydrocarbon chain at C-17. It is significant that these are the same conditions required for optimum growth of a sterol-requiring organism such as Sacch. cerevisiae when grown anaerobically (Proudlock et al., 1968).

A disadvantage of n.m.r. and e.s.r. studies is that the presence of the label (paramagnetic groups for e.s.r. or isotopic substituent for n.m.r.) introduces to the system an unnatural molecule which could cause perturbations in the system, especially where there is close packing of molecules (Darke et al., 1971; Lapper et al., 1972).

X-Ray diffraction studies of phospholipid bilayers indicate that hydration decreases their thickness and increases the average surface area of the lipid molecules (Levine and Wilkins, 1971). This finding suggests greater mobility of the fatty-acyl residues, a suggestion which is supported by the observation of a diminished phase-transition temperature when hydrated (Williams and Chapman, 1970). On addition of cholesterol to dipalmitoyllecithin at 25°C, more water is taken up (Ladbroke et al., 1968). Cholesterol at 7.5 mole % does not disturb the packing of the chains, but causes the fatty-acyl

chains to become ordered perpendicularly to the plane of the bilayer. Addition of more cholesterol up to 50 mole % causes fluidization of the chain. This finding is consistent with evidence from e.s.r. (Oldfield and Chapman, 1971) and differential scanning calorimetry (d.s.c.) studies (Ladbroke et al., 1968). Cholesterol increases the thickness of the bilayer and decreases the average surface area occupied by each phospholipid molecule in liposomes (Levine and Wilkins, 1971). This discovery suggests a restriction of motion of the fatty-acyl residues.

Thermal studies are useful for examining the phase change from crystalline to liquid-crystalline states. The temperature of the phase transition depends on the phospholipid structure, more particularly of the fatty-acyl residues and the nature of the polar groups, and on the degree of hydration and also the presence of a sterol. If the head group and degree of hydration are the same, then a lipid with a larger fatty-acyl chain has a higher transition temperature than one with a shorter one (Chapman et al., 1967); one with an unsaturated fatty-acyl residue has a lower transition temperature than with a saturated one (Ladbroke et al., 1968), and lipid with a cis unsaturated fatty-acyl chain has a lower transition temperature than when the bond is trans (Chapman et al., 1966). With regard to polar groups, dimyristoyl phosphatidylethanolamine has a transition temperature of 48°C, some 25°C above that of dimyristoyl phosphatidylcholine (quoted in Oldfield and Chapman, 1972b).

A mixture of the two lipids has a broad transition temperature range, which suggests there are clusters of crystalline and liquid-crystalline lipids in the bilayer.

The effect of water is also important. Differential scanning calorimetry indicates there are ten moles of water per mole of lecithin (Williams and Chapman, 1970). The presence of this water lowers the transition temperature (Williams and Chapman, 1970). However, concentrations of water greater than 10 moles % do not cause a further change in the transition temperature (Chapman et al., 1967).

Additions of cholesterol above a concentration of 20 mole % to dipalmitoyllecithin in water lowers the transition temperature, and decreases the heat of transition (Ladbroke et al., 1968). At 50 mole % the transition temperature is abolished resulting in a state of intermediate fluidity, and Ladbroke et al. (1968) suggested that cholesterol controls the fluidity by disruption of the crystalline packing below the transition temperature, and by inhibiting flexibility of the fatty-acyl chains in the liquid-crystalline phase. This suggestion is consistent with the data obtained by n.m.r. and e.s.r. studies. Similar results were obtained by d.s.c. on a mixture of sphingolipids and cholesterol (Oldfield and Chapman, 1972a).

Sterol structure has been shown to be important in phase transition. de Kruyff et al. (1972) showed that incorporation of cholesterol causes a large decrease in the phase

transition temperature of isolated membranes of Acholeplasma laidlawii; 32 mole % abolishes the transition with synthetic 1'-oleoyl-2-stearoyl-sn-glycero-3-phosphoryl-choline. However epicholesterol, which has a 3α-hydroxyl group, and a sterol without a side chain at C-17 or the 3β-hydroxyl group replaced by a 3-keto group, did not exhibit any effect, de Kruyff et al. (1973b) used d.s.c. to study the effect of cholesterol (33.3 mole %) on the phase-transition temperatures of liposomes made from chemical derivatives of phospholipids, and found that, for any change to be manifested, there was no requirement for the oxygen atom at the acyl ester link. Using mono-glucosyldiacylglycerol, they showed that the phosphoryl base was not required. They emphasised the importance of the 3β-hydroxyl groups on the sterol, and suggested it to be important for interaction with phospholipids (cf also Darke et al., 1971). The same workers (de Kruyff et al., 1973b) also found that, in an equimolar mixture of dioleoyllecithin and distearoyllecithin, the cholesterol (25 mole %) preferentially associated with the unsaturated lecithin. The sterol associated with both phospholipids only at high temperatures. Using d.s.c. de Kruyff et al. (1974) found that cholesterol would mix randomly with lecithins possessing fatty-acyl chains which differ by two carbon atoms, but that, if the difference was four or more carbon atoms, then cholesterol would preferentially associate with the fatty-acyl chains with the lower phase-transition temperature.

Lipids from erythrocyte membranes do not have a phase-transition temperature, but removal of cholesterol gives rise to two transitions the lower of which is attributable to highly unsaturated species of fatty-acyl chain (Williams and Chapman, 1970). Adding back cholesterol to the lipid mixture removes both transitions, which is further evidence to support the dual role of cholesterol to 'rigidize' and 'fluidize' regions of the membrane.

When applied as a monolayer at a water-air interface, lipids can be compressed to give a minimum surface area per molecule. Addition of a sterol will give an average molecular surface area which is less than that expected if there was no interaction (Leathes, 1925). This phenomenon is called 'condensation', and its extent gives more information about the type of interaction between phospholipids and sterols.

The condensation effect is known to depend on the structure of both the phospholipids and the sterols. Using different steroids, Demel et al. (1972a) found the most effective condensation to be with cholesterol, and rather less condensation with ergosterol or stigmasterol. No condensation was obtained with a 3α-hydroxysterol, a ketosteroid, or if a side chain at C-17 was absent. These workers suggested that the side chain was important with particular reference to the double bond at C-22 and substituents at C-24 encountered in the plant sterols. This is in contrast to results obtained by Ghosh and Tinoco (1972)

who found that cholesterol and β-sitosterol gave similar condensation results with synthetic lecithins, but different from the effects obtained with dihydrocholesterol or ergosterol. Ghosh and Tinoco (1972) proposed that the degree of unsaturation in ring B of the sterol nucleus was more important than the structure of the side chain.

The nature of the fatty-acyl residues has also been examined. Condensation occurs with cholesterol if the phospholipid contains one saturated and one unsaturated fatty-acyl residue, though the position on the glycerol residue was not significant. Condensation did not occur if both chains were polyunsaturated (Demel et al., 1972c).

The presence of an oxygen atom at the acyl ester linkage at C-2 of glycerol, or between the phosphorus and carbon atoms at C-3 or the presence of the phosphoryl-base was not required for condensation (de Kruyff et al., 1973b). This discovery coincided with the finding that these structural features had no influence on the phase-transition temperatures. Ghosh et al. (1973a) found that cholesterol also condensed with phosphatidylethanolamine.

The finding that, for maximum interaction with a phospholipid to be manifested, the sterol should have a planar nucleus and an alkyl side chain (Butler et al., 1970; Hsia et al., 1972; Demel et al., 1972a) indicated the importance of hydrophobic interaction. From their studies

of the condensing effect of cholesterol on monolayers of various lecithins, Ghosh et al. (1973b) suggested that the greatest interaction was obtained if the first nine atoms of the acyl residue were saturated, and suggested an interaction between the nucleus of the sterol and these atoms. This finding is in agreement with the existence of a complex suggested from e.s.r. and n.m.r. evidence (Hubbell and McConnell, 1971; Darke et al., 1971). However the necessity for the 3β rather than the 3α hydroxyl group on the sterol does not suggest the presence of polar interactions, but does indicate the possibility of the importance of hydration (de Kruyff et al., 1973b).

Rothman and Engelman (1972) have proposed a model to describe the interaction between sterols and phospholipids, in which the molar ratio of the two compounds is not important, and in which steric interactions play a prominent part. The 'intermediate fluid' condition is explained by the close packing of the carboxyl end of the fatty-acyl residues and the nucleus of the sterol, but the loose packing can be explained by an interaction of the terminal end of the fatty-acyl residues and the sterol side chain. The freedom of the terminal fatty-acyl residues is also enhanced by the mobility of the sterol side chain.

The relationship between lipid structure and permeability has been examined for synthetic liposomes and also natural

membrane lipids. The permeability of liposomes to solutes depends on the nature of the polar groups on the phospholipid, as well as on the chain length and number, position and geometry of the unsaturated fatty-acyl residues in the lipids. The chemical structure and configuration of the sterol, and the phospholipid-sterol molar ratio also influence liposome permeability (van Deenen, 1972).

Liposomes composed principally of lecithin have decreased permeability to glucose in the presence of cholesterol, though this also depends on the nature of the fatty-acyl residues. Cholesterol had more effect on liposomes derived from 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoryl-lecithin than if both fatty-acyl residues were poly-unsaturated (Demel et al., 1968). This finding is in agreement with the condensing properties of cholesterol and lecithins containing different fatty-acyl residues (Demel et al., 1972c). Similarly a greater degree of unsaturation in the fatty-acyl residues increased the permeability of liposomes to glycerol and erythritol. A shorter chain length or combination of acyl chains with widely different chain lengths also increased permeability. Permeability was lowered in the presence of cholesterol proportionally to the amount of cholesterol present (de Gier et al., 1968). The diminished permeability in the presence of cholesterol in various liposomes has been demonstrated only when there is a condensing effect between cholesterol and the corresponding lecithin (Demel et al.,

1972c).

The nature of the sterol structure which affects the permeability of egg-yolk lecithin liposomes has been studied (Demel et al., 1972b). Cholesterol, cholestanol and 7-dehydrocholesterol all caused a greater decrease in permeability than did ergosterol or stigmasterol. No decrease was observed for epicholesterol, 3-keto-steroids, coprostanol or a sterol without an alkyl side chain at C-17. Demel et al. (1972b) suggested that, in order to induce a lowering of permeability, a sterol must have a 3β-hydroxy group, a planar nucleus and a side chain at C-17. These requirements are identical with those deduced from other types of experiment.

Permeability studies on intact membranes from Acholeplasma laidlawii with different sterol compositions have been reported. As with liposomes, cholesterol caused lowered permeability to glucose, but this effect was not observed with epicholesterol (de Kruyff et al., 1972). Flux of ^{14}C erythritol through membranes of Acholeplasma laidlawii, the composition of whose lipids is manipulated by supplementary different fatty acids and sterols in the medium, was found to be higher in the order: linoleic acid > oleic acid > eloidic acid (trans 18:1) > stearic acid. All types of membrane exhibited lowered permeability in the presence of cholesterol or ergosterol, but not with epicholesterol or coprostanol (de Kruyff et al., 1973a).

Huang et al. (1974) grew Acholeplasma laidlawii in the presence of arachadic acid ($C_{20:0}$) or lauric acid ($C_{12:0}$) and obtained corresponding enrichments in the fatty-acyl chains of the phospholipids. Cells enriched with the longer-chain acid were less permeable to glycerol, were more sensitive to osmotic shock, and were less mobile when measured by e.s.r. compared to those cells enriched in the shorter acids. They also measured the thickness of such membranes by electron microscopy and found there was no difference; they suggested that the apparent restricted motion of the larger fatty-acyl residues was due to the tighter packing as they occupied the same volume as shorter residues.

The data on phospholipid-sterol interactions suggest that the function of a sterol is to regulate the permeability of membranes by controlling the degree of fluidity, and giving stability over a wide range of temperature by allowing a certain latitude of fatty-acyl structure in the phospholipids (Ladbroke et al., 1968; Oldfield and Chapman, 1972a; Demel et al., 1972c). This suggestion is supported by the presence of crystalline and liquid-crystalline phases in membrane lipids, and the dual role of cholesterol (Darke et al., 1971; Oldfield and Chapman, 1971; Williams and Chapman, 1970). More recent support comes from the observation by Rottem et al. (1973a) that Mycoplasma mycoides will not survive below

25°C if adapted to grow in the absence of supplemented cholesterol, when the membrane lipids would be crystalline. Polyene-resistant mutants of Sacch. cerevisiae, which have altered sterol compositions, were also found to die if stored at 4°C (Karunkaran and Johnson, 1974). They suggested that death could be due to leakage of essential ions, caused by altered permeability. Also of interest is that changes in both crystalline and liquid-crystalline phases are observed by d.s.c. (Steim et al., 1969), and X-ray diffraction (Engelman, 1971) studies on Acholeplasma laidlawii which have membranes with low levels of cholesterol.

The presence of sterol in a membrane also appears to regulate the activity of certain enzymes. Rottem et al. (1973) observed a reversible phase change, by d.s.c. and a break in the Arrhenius plot of ATPase activity versus temperature, in the membranes of Mycoplasma mycoides, if adapted to grow in cholesterol-deficient medium, but not in cells containing the full complement of cholesterol. Cobon and Haslam (1973) made similar observations using Sacch. cerevisiae grown anaerobically with a range of ergosterol supplements. It appears that a 3β -hydroxyl group on the sterol is important, as epicholesterol incorporated into the membranes of Acholeplasma laidlawii caused a break in the Arrhenius plot of ATPase activity, though this did not occur if cholesterol was provided in the medium (de Kruyff et al., 1973c).

Sterol-Protein Interactions

The interaction between 'Folch-Lees' apoprotein and a variety of steroids was examined by London et al. (1974). They found the greatest affinity between hydrophobic protein and cholesterol and, to a lesser extent, 7-dehydrocholesterol. The presence of an α-hydroxyl group or keto group at C-3 of the steroid nucleus, a non-planar steroid nucleus such as coprostanol, or the absence of a side chain at C-17 resulted in less efficient interaction. These findings are comparable with the steroid structural features required for optimum sterol-phospholipid interactions. London et al. (1974) pointed out the importance of the orientation of the hydroxyl group of the sterol, and also suggested that the presence of the hydrophobic side chain at C-17 is important in hydrophobic interactions.

OSMOTIC LYSIS

The behaviour of plasma membranes may be observed in an undifferentiated eukaryotic system, such as that found in yeast, by using sphaeroplasts prepared after removal of cell walls. Properties which can be examined include osmotic lysis and extensibility of the plasma membrane. Information obtained gives clues about membrane structure and the molecular basis of its activities.

Resistance to lysis of sphaeroplasts from Sacch. carlsbergensis, in response to osmotic shock, has been shown to be lowered by the presence of chelating agents, but this effect was inhibited by K^+, Na^+, Mg^{2+} or spermidine (Indge, 1968). Indge (1968) suggested that there is a cation-binding site in the membrane which confers stability on the membrane. Corner and Marquis (1969) examined the effect of various molecules as osmotic stabilisers for protoplasts of Bacillus megatherium, and found that the porosity of the membrane to these molecules increased during osmotic swelling, by slowly diluting the stabiliser. The membranes were found to be highly extensible in hypotonic solutions, eventually stretching sufficiently to allow solute to enter the protoplast through pores, hence leading to rupture by brittle fracture.

Alterthum and Rose (1973) grew Sacch. cerevisiae NCYC 366 anaerobically in a defined medium supplemented with ergosterol and either oleic, linoleic or γ-linolenic acid; the fatty acids supplied accounted for 54 - 65% of the fatty-acyl residues of the cells. The resistances to osmotic lysis of sphaeroplasts obtained from these cells by the action of a basidiomycete glucanase were compared. These workers showed that sphaeroplasts from cells grown in a medium supplemented with oleic acid were more resistant than those from linoleic acid-supplemented medium, while those grown in the presence of linolenic acid were unstable even in isotonic conditions, unless spermine was included

in the glucanase digest. They attributed the lowering of stability of sphaeroplasts, as the degree of unsaturation of the fatty-acyl residues was increased, to the diminished stability in the plasma membrane caused by the perturbation of the close packing of the fatty-acyl chains of the phospholipids.

The work reported in this thesis is in many ways an extension of that described by Alterthum and Rose (1973). Sphaeroplasts were obtained from Sacch. cerevisiae N.C.Y.C. 366, grown anaerobically in the presence of different sterols and Tween 80 (a source of $C_{18:1}$ acid), and an examination made of certain physical properties of the plasma membranes of the sphaeroplasts.

METHODS AND MATERIALS

METHODS

Organism

The yeast used in this study was Sacch. cerevisiae N.C.Y.C. 366, a strain which can rapidly be converted into sphaeroplasts (Eddy and Rudin, 1958). The yeast was maintained on slopes of glucose-salts-vitamins medium (Diamond and Rose, 1970), stored at 4°C and subcultured at weekly intervals.

Experimental cultures

The yeast was grown under strictly anaerobic conditions which induce a nutritional requirement for a sterol and an unsaturated fatty acid (Andreasen and Stier, 1954). The medium contained per litre: 100g glucose, 3.0g $(NH_4)_2SO_4$, 3.0g KH_2PO_4, 2.0g yeast extract (Oxoid), 25mg $MgSO_4.7H_2O$, 25mg $CaCl_2.2H_2O$, 2.0g Tween 80 (as a source of $C_{18:1}$ acid), and 5.0mg of the sterol indicated. The desired sterol was added as a solution (2.5ml) in 95% (v/v) ethanol. Cultures (1l) in 2 l round flat-bottomed flasks were inoculated and grown as described by Alterthum and Rose (1973) except that the control medium was supplemented with Tween 80, but not with sterol (Fig. 3). Growth was followed by removing portions of the culture

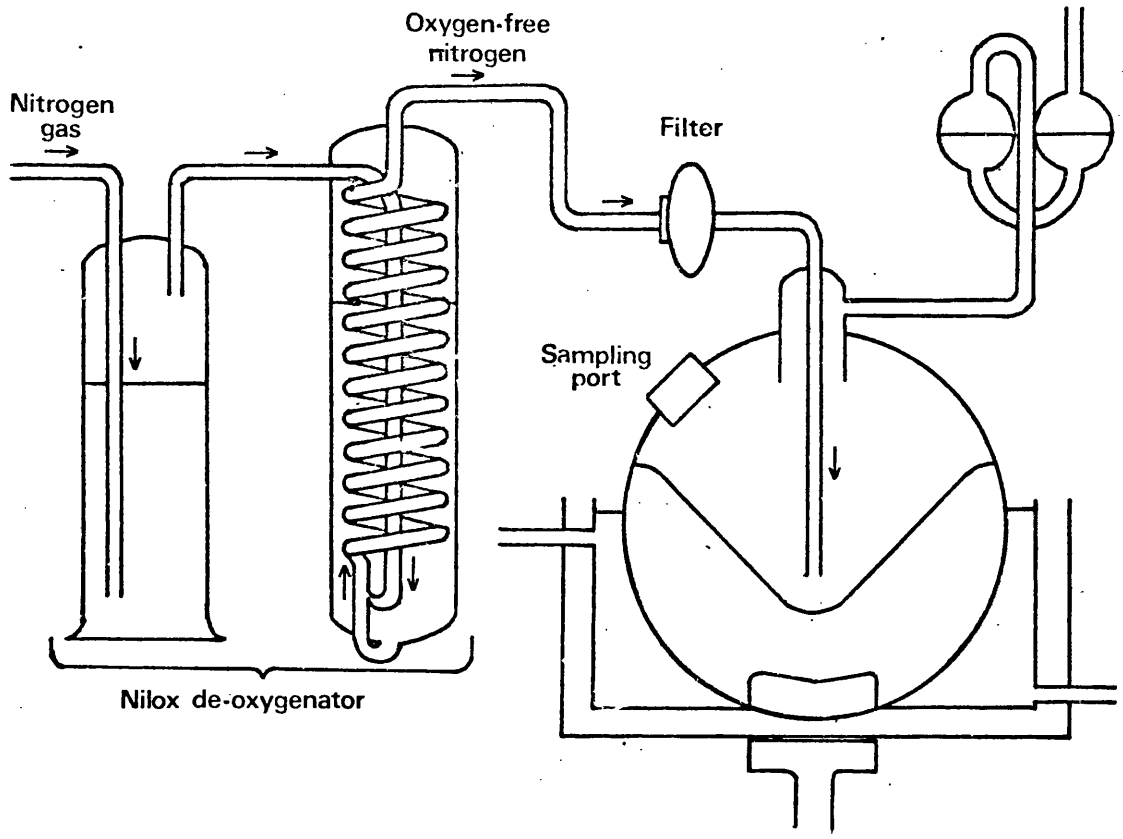


Fig.3.Apparatus used for anaerobic cultivation of Saccharomyces cerevisiae NCYC 366

with a hypodermic syringe through a Suba seal port fitted on the side of the flask. Cultures were harvested when the density reached 0.22 - 0.24mg dry wt. per ml. Before harvesting, 2 ml of a solution containing 10mg each of chloramphenicol and cycloheximide were injected into the flask through the Suba seal, and the culture incubated for a further 15 min. The culture was then centrifuged in a M.S.E. High Speed '18' refrigerated centrifuge at 4°C for 5 min at 12,250 x g. Cells which were to be analysed for lipid or sterol content or composition were washed twice with water at 4°C, freeze dried, and stored at -20°C over silica gel. Cells which were to be converted into sphaeroplasts were washed twice with 1.2M sorbitol containing 10mM $MgCl_2$ and 10mM imidazole hydrochloride (pH 6.0).

Extraction and estimation of total lipids

Lipids were extracted from freeze dried cells by a modification of the method of Letters (1968b) with the exception that, during chloroform-methanol extraction of the residue from the ethanol extraction, the residue was first suspended in methanol for 10 min to disperse the cells before addition of chloroform. Other modifications adopted have already been described (Hunter and Rose, 1972). Total lipid estimations were carried out gravimetrically as described previously, the desiccator being evacuated for at least 48 h before the pans were

weighed to constant weight.

Analysis of phospholipids

Total phospholipid in lipid extracts was assayed as described by Hunter and Rose (1972), using the method of Chen et al. (1956). Individual phospholipids were separated by thin-layer chromatography using several solvent systems, and identified using authentic samples of phospholipids and specific reagents (Watner et al., 1961; Skidmore and Enteman, 1962; Dittmer and Lester, 1964). The distribution of phospholipids in extracts was determined by using two-dimensional thin-layer chromatography. Plates (20cm x 20cm) were coated with a layer (0.25mm) of Kieselgel HF_{254} (Merck), washed in chloroform, and activated at 105°C for 30 min then cooled over a desiccant before use. They were then developed in the first direction in chloroform-methanol-ammonia (0.88 sp. gr.) (65:42.5:6.5, v/v/v) and, after drying in air for 20 - 30 min, in the second direction with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:4, v/v/v/v/v). Both solvent systems contained butylated hydroxytoluene (0.005% w/v) (Neudoeffer and Lea, 1966).

Phospholipids spots were detected by exposing the plate to iodine vapour. Phospholipids were eluted from the silica gel with two portions (3ml) of chloroform-methanol-water (5:5:1, v/v/v) followed by 3ml of methanol, and 3ml of

methanol-acetic acid-water (95:1:5, v/v/v). Phospholipid was determined by assaying the phosphorus content of the extract using the method of Chen et al. (1956). A small portion of silica gel was also removed from each plate, and eluted for use as a blank. Values for phosphorus contents were multiplied by 25 to give the total phospholipid content, which is expressed as mg per 100mg dry wt. cells.

Analysis of non-polar lipids

Non-polar lipids were separated by quantitative thin-layer chromatography on plates coated with a layer (0.4mm) of Kieselgel $HF_{254 + 366}$ (Merck); the plates were not activated before use. Plates were developed with petroleum spirit (40-60°C)-diethyl ether-acetic acid (70:30:2, v/v/v). The solvent system contained butylated hydroxytoluene (0.005%, w/v). Non-polar lipids were identified using ultraviolet illumination at 254 nm for compounds containing unsaturated acyl residues, and 366 nm for sterols and sterol esters, using appropriate standards.

Triacylglycerols were eluted from the silica gel with two portions of chloroform (3ml) followed by two portions of diethylether (3ml). The first elution with diethylether was carried out by shaking the suspension in a Rotary Evapomix for 10 min prior to centrifugation. Sterols and sterol esters were eluted with three portions (3ml)

of chloroform-methanol (4:1, v/v). Triacylglycerols were assayed by determining the total content of fatty-acyl residues, as their methyl esters, using gas-liquid chromatography. Heptadecanoic acid was used as an internal standard. Both fast-reacting (5, 7-diene) and slow-reacting sterols were assayed by the method of Moore and Baumann (1952). Sterol contents were calculated as fast- and slow-reacting sterols, respectively, from a standard curve prepared using ergosterol or cholesterol. Sterol esters were saponified and assayed as described by Hunter and Rose (1972).

Squalene was separated by thin layer chromatography on plates coated with Kieselgel HF_{254}, and developed in petroleum spirit (40-60°C). Squalene was identified by u.v. radiation against a standard of squalene run simultaneously. Elution was with petroleum spirit (40-60°C) and squalene was assayed by the method of Trappe (1938).

Preparation of fatty-acid methyl esters

Phospholipids and neutral lipids were separated by thin-layer chromatography as already described. Neutral lipids were eluted from the silica gel with diethylether (100ml) followed by chloroform (100ml). Phospholipids were eluted as already described. Extracts were evaporated to dryness, and methyl esters of the fatty-acyl residues

prepared as described by Hunter and Rose (1972).

Preparation of sterol derivatives

Sterol acetates were prepared by dissolving purified sterol (1mg) in dry pyridine (0.5ml) and adding acetic anhydride (0.5ml) (Kuksis, 1967). The reaction mixture was left at room temperature overnight. The excess reagent was then evaporated off under a stream of nitrogen gas, and the acetates taken up in a small volume of chloroform.

Saponification of sterol esters prior to acetate formation was carried out as already described (Hunter and Rose, 1972).

Trimethylsilyl ethers of sterols separated by thin-layer chromatography were prepared by dissolving the sterol (1mg) in dry pyridine (1ml) and adding 0.2ml hexamethyl disilizane and 0.1ml triethylchlorosilane (Kuksis, 1967). The mixture was shaken and left for 15 min, then partitioned between water and petroleum spirit (40-60°C). The petroleum layer was separated, and the mixture re-extracted with two further portions (1ml) of petroleum spirit. The extract was evaporated to dryness and redissolved in chloroform.

Gas-liquid chromatography

All samples were assayed using a Pye 104 model 64 unit with flame ionization detectors.

Free sterols were separated on 3% OV-1 supported on 100-120 mesh Gas Chrom Q packed in a stainless-steel column (5ft x 0.25 inch). The column was maintained at 225°C with a nitrogen gas flow rate of 30ml/min and the detector at 250°C. Sterol acetates and trimethylsilylethers were separated on 3% OV-17 supported on 100-120 mesh Gas Chrom Q packed in a stainless-steel column (9ft x 0.25 inch). The column was maintained at 255°C with a nitrogen gas flow rate of 70ml/min, and the detector at 300°C. Retention times, relative to cholestane, were determined for standard sterols and sterol acetates.

Fatty-acid methyl esters were separated on 15% EGSS-X supported on 100-120 mesh Gas Chrom P. The EGSS-X was packed in a stainless-steel column (5ft x 0.25 inch). The column was maintained at 175°C with a nitrogen gas flow rate of 50ml/min and the detector at 200°C. Retention times, relative to methyl palmitate, were determined for standard mixtures of fatty-acid methyl esters.

All samples were injected onto the column in 1μl chloroform. Peak areas were measured, and the relative amounts of compounds calculated by the method of Pecsok

(1959).

Preparation of sphaeroplasts

Sphaeroplasts were prepared from freshly harvested cells by digestion with a glucanase from Basidiomycete QM 806. The method used was a modification of that described by Alterthum and Rose (1973) as described by Cartledge and Rose (1973). Glass vessels and centrifuge tubes used in the preparation of sphaeroplasts were flushed with high-purity nitrogen gas before use. Throughout the preparation, chloramphenicol and cycloheximide were included in all reaction mixtures and buffers each at about 0.2mg/ml.

Isolation of plasma membranes

Purified preparations of plasma membranes from Sacch. cerevisiae N.C.Y.C. 366 were obtained by labelling intact sphaeroplasts by iodination (Phillips and Morrison, 1970; Marchalonis et al., 1971), lysing the sphaeroplasts, and isolating plasma membranes by density-gradient centrifugation. Iodination was carried out by a modification of the method described by Shibeci et al. (1973). The reaction mixture (50ml) consisted of a suspension of sphaeroplasts (from 800mg dry wt cells) in 10mM imidazole buffer (pH 6.5) containing 1.2M sorbitol, 2mM $MgCl_2$, 1.0×10^{-7}M lactoperoxidase, and 0.1 Ci $Na^{125}I$/ml. Iodination was

initiated by adding H_2O_2 to a concentration of 8μM. Equal amounts of H_2O_2 were added at one-minute intervals up to 5 min. The sphaeroplasts were then removed by centrifugation, washed four times with buffered 1.2M sorbitol containing 10mM $MgCl_2$, and lysed by a combination of osmotic and gentle mechanical shock. A suspension of the sphaeroplasts in 8% (w/v) sucrose was submitted to ten strokes in a Teflon-glass hand homogenisor (0.1mm clearance). The lysate was centrifuged at 3,000 x g for 10 min, and the membrane-containing pellet washed three times with 8% (w/v) sucrose (about 20ml). The pellet was then suspended in 15ml 8% (w/v) sucrose, and portions (3ml) of the suspension layered on the top of a discontinuous gradient of sucrose solutions each of which was made up in 10mM tris buffer (pH 7.4). The gradient consisted of solutions containing 4ml each of 10, 20, 30, 40, 50, 57.5 and 62% (w/v), sucrose. An overlay of 2ml tris buffer (pH 7.4) was layered on top of the sample. The tubes containing the gradients were centrifuged at 26,250 x g for 16 h at 4°C. Visible bands in the gradient were then removed with a hypodermic syringe. After removing the remaining supernatant, the pellet was resuspended in a small volume of water. The concentration of sucrose in each fraction removed from the gradient was measured with a refractometer and the refractometer readings converted to sucrose density by a standard curve taken from the data of de Duve *et al.* (1959). Absorbancies of the fractions at 280nm were measured in

a Unicam S.P. 1800 spectrophotometer using cells of 1cm light path. Portions (1ml) of each fraction from the gradient, as well as of washings of the sphaeroplasts and of the supernatant and washings of the centrifuged membrane-containing pellet obtained from the sphaeroplast lysate, were added to 5 - 10ml of scintillation liquid (Unisolve, Koch-Light Ltd.) in 20ml vials. The radioactivity of the contents of the vials was counted for 20 min in a Beckman scintillation counter (model CPM 500). Preparations of plasma membrane, located as described in the Results section, were freeze dried, and stored at -20°C.

Assessment of osmotic fragility

The susceptibility of sphaeroplasts to osmotic lysis was measured at 20°C by adding 0.1ml portions of sphaeroplast suspensions (containing 10mg dry wt. equiv./ml) to 2.9ml portions of 10mM imidazole buffer (pH 6.5) containing 2mM $MgCl_2$ and sorbitol at concentrations ranging from 1.2 to 0.0M. After adding the sphaeroplast suspension, the contents of each tube were gently shaken, left for 10 min, and the absorbance (E^{600}_{1cm}) of the suspension measured.

Measurement of sphaeroplast volumes

Volumes of cells and sphaeroplasts were measured as

described by Harvey (1968) and Kubitschek (1969) using an electronic particle counter (Electrozone-Celloscope; model 111 LTS; Particle Data Inc., Elmhurst, Illinois, U.S.A.). The counter was fitted with a 60 μm-diameter orifice, and was calibrated with standard latex spheres (2.03 and 9.79 μm-diameter; Coulter Electronics, Luton, Bedfordshire, England). Signals from the counter were sized in a multichannel pulse-height analyser (Nuclear Data Inc., Palatine, Illinois, U.S.A.; series 1100) and the volume distribution in the suspension of cells or sphaeroplasts plotted on a X-Y plotter (Hewlett-Packard Inc., Pasadena, California, U.S.A.; model 7035B). Suspensions of cells in 0.2M NaCl, or of sphaeroplasts in buffered sorbitol of the molarity indicated and supplemented with 0.2M NaCl, were diluted to about 10^4 particles/ml to avoid coincidence counting, and the suspension drawn through the counter orifice at 5m/sec until the analyser had assembled sufficient data to present a reasonable analysis. Suspensions of cells were submitted to ultrasonic vibration (30 sec at 0°C) with a M.S.E.-Mullard instrument to separate individual cells from clumps. All measurements were made at 21°C.

Force-area studies on dispersed monolayers

These were carried cut by methods similar to those described by Gaines (1966). Solutions of cholesterol or ergosterol

and egg-yolk lecithin, containing between 0.6 and 1.0μg perμl, were prepared using the spreading solvent mixtures of chloroform:methanol (2:1, v/v). Portions (50μl) of these solutions or mixtures containing different molar ratios of one of the sterols and lecithin were applied to the surface of a Langmuir trough (Unilever, Welwyn, Herts.) over an area of 558cm^2. The trough had previously been cleaned by suction to remove all grease and filled with glass-distilled water (pH 6.0) which was also cleaned to remove surface-active impurities, this being repeated several times until there was no change in surface pressure recorded. The trough was filled so as to form a high meniscus, care being taken to avoid leakage.

Mica slides (1cm wide) were used and the force due to the surface pressure was measured as the barriers were brought together at a rate of 11cm/min, the final area bounded by the barriers being 46.5cm^2. The temperature was kept constant at 30°C.

Plots were made of force due to surface pressure (expressed as dynes/cm) against the mean area per lipid molecule at the air-water interface. The area per lipid molecule was calculated as follows:

No. of molecules applied to trough =

$$\frac{\text{wt. of lipid } (\mu g)}{\text{mol.wt.} \times 10^6} \times 6.02 \times 10^{23}$$

Area per lipid molecule ($Å^2$) =

$$\frac{\text{area of trough } (cm^2) \times 10^{16}}{\text{no. of molecules}}$$

For a quantitative evaluation of the interactions the variations of the mean molecular areas at a pressure of 5 dynes/cm were measured and plotted against the mole % of the phospholipid:sterol mixtures.

Similarly mixtures of either cholesterol or ergosterol and phosphatidyl-myo-inositol, dissolved in benzene:chloroform (2:1 v/v) were applied to the trough and the capacities of the sterols to decrease the average molecular area of the phospholipid were measured.

MATERIALS

Campesterol (cholest-5-en-24(R)-methyl-3β-ol), 7-dehydrocholesterol (cholest-5,7-dien-3β-ol), β-sitosterol (cholest-5-en-24(R)-ethyl-3β-ol), and stigmasterol (cholest-5,22-dien-24(S)-ethyl-3β-ol) were obtained from Applied Science, State College, Pa., U.S.A. Cholesterol (cholest-5-en-3β-ol) and ergosterol (cholest-5,7,22-trien-24(R)-methyl-3β-ol) were supplied by Sigma Chemical Co. (London). A small quantity of 22,23-dihydrobrassicasterol (cholest-5-en-24(S)-methyl-3β-ol) was

kindly supplied by Dr. H.W. Kircher, Department of Agricultural Biochemistry, University of Arizona, Tucson, Arizona, U.S.A. All of the sterols were shown by gas-liquid chromatography to be 97-99% pure. They were used without further purification.

All other chemicals used were AnalaR or of the highest purity available commercially. Chloroform, ethanol and methanol were redistilled before use. Sigma Chemical Co. (London) were the suppliers of cholesterol palmitate, dipalmitin, heptadecanoic acid, monopalmitin, oleic acid, phosphatidylcholine, phosphatidylethanolamine, squalene and lactoperoxidase. Egg-yolk lecithin and phosphatidyl-myo-inositol were obtained from Lipid Products (Nutfield, Redhill, Surrey). Applied Science provided mixtures of fatty acid methyl esters, and 15% EGSS-X on Gas Chrom P, while 3% OV-17 and 3% OV-1, each on Gas Chrom Q, came from Phase Separations, Rock Ferry, Cheshire, England. Silica gels (Merck) for thin-layer chromatography were supplied by Anderman and Co., Tooley St., London, England, Tween 80 and Unisolve by Koch-Light Laboratories, Colnbrook, Bucks, England, and glycerol palmitate by British Drug Houses, Poole, Dorset, England. $Na^{125}I$ for membrane iodination was obtained from the Radiochemical Centre, Amersham, Bucks. Beta-Glucanase from Basidiomycete QM 806 (Huotari et al., 1968) was prepared and purified as described by Alterthum and Rose (1973).

RESULTS

Growth of cells in the presence of different sterols

Saccharomyces cerevisiae N.C.Y.C. 366 was grown anaerobically in medium supplemented with various amounts of ergosterol. The rates of growth in the presence of 20mg per litre or 5mg per litre were similar, but greater than in the presence of 3mg per litre (Table 1). For subsequent experiments a concentration of 5mg of sterol per litre was used.

Cultures of the yeast grown anaerobically in the presence of campesterol, cholesterol, 7-dehydrocholesterol, 22,23-dihydrobrassicasterol, ergosterol, β-sitosterol or stigmasterol did not differ in the duration of the lag phase of growth or in the rate of exponential growth. Moreover, cells from mid-exponential phase cultures containing any of these sterols did not differ in size as judged by microscopic examination. Electronic cell size measurements of the diameters of cells and sphaeroplasts of Sacch. cerevisiae N.C.Y.C. 366 grown anaerobically in the presence of cholesterol or ergosterol indicate that there is no significant difference between them (Table 2).

Lipid content and composition of cells

Analyses of the free and esterified sterol fractions of cells grown in media containing any one of seven sterols

TABLE 1

GROWTH RATES OF Saccharomyces cerevisiae N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF ERGOSTEROL.

Concentration of ergosterol (mg/litre)	Growth rate per hour during exponential phase
1	0.35
3	0.40
5	0.75
10	0.78
20	0.73

TABLE 2

DIAMETERS OF WHOLE CELLS AND SPHAEROPLASTS OF *Saccharomyces cerevisiae* N.C.Y.C. 366GROWN ANAEROBICALLY IN THE PRESENCE OF CHOLESTEROL OR ERGOSTEROL

Sterol included in medium	Diameter (μm)	
	Whole Cells	Sphaeroplasts
Cholesterol	7.25 ± 1.02 (5)	5.46 ± 0.52 (5)
Ergosterol	6.86 ± 0.46 (7)	4.98 ± 0.94 (4)

Figures in parentheses indicate the number of analyses conducted on independently-grown batches of cells. The values are quoted ± 95% confidence limits.

(Tables 3 and 4) show that the free-sterol fraction is enriched with the sterol supplied in the medium. The extent of enrichment ranges from 67% in cells grown in 7-dehydrocholesterol-containing medium to 93% in cells grown in the presence of stigmasterol. Most of the remaining sterol was accounted for by a tetraethenoid sterol 24(28)-dehydro-ergosterol and ergosterol which are the two principal sterols synthesized endogenously by aerobically grown cells (Hunter and Rose, 1972). However, the sterol-ester fraction was not always enriched to the same extent (Table 4). With cells grown in the presence of ergosterol only 39% of the esterified sterol was ergosterol but, in cells grown in the presence of campesterol, over 91% of the sterol-ester fraction contains campesterol.

Although inclusion of different sterols in the medium led to appreciable enrichment of the free-sterol fraction with the sterol supplied, it was imperative to establish that the contents and composition of other lipids in the cells did not differ when the nature of the sterol in the medium was changed. The total lipid content, and the contents of the major classes of lipid, in cells grown in the presence of each of six sterols are shown in Table 5. Because of the small amount of sterol available, analyses were not made on lipids from cells enriched in 22,23-dihydrobrassicasterol. The total lipid contents of cells varied little, with the exception of cells grown

TABLE 3

COMPOSITION OF THE FREE STEROLS OF *Saccharomyces cerevisiae* N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF DIFFERENT STEROLS

Sterols included in medium	Percentage composition of the free sterols			
	Sterol included in medium	Zymosterol	Ergosterol	24(28)-Dehydro-ergosterol
Campesterol (4)	92.3	0.7	-	7.0
Cholesterol (5)	74.9	-	7.4	17.0
7-Dehydro-cholesterol (4)	66.6	7.1	12.2	13.0
22,23-Dihydro-brassicasterol (4)	76.8	5.9	-	17.3
Ergocterol (5)	80.0	3.5		16.4
β-Sitosterol (4)	83.8	1.1	15.0	-
Stigmasterol (4)	92.6	2.0	1.4	3.8

Figures in parentheses indicate the number of analyses Conducted on independently-grown batches of cells. The 95% confidence limits of the values for the sterols supplemented to the medium ranged from ± 17.7 for ergosterol-supplemented cells to ± 0.5 for cells grown in the presence of β-sitosterol.

TABLE 4

COMPOSITION OF THE ESTERIFIED STEROLS OF *Saccharomyces cerevisiae* N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF DIFFERENT STEROLS

Sterol included in medium	Percentage composition of the esterified sterols			
	Sterol included in medium	Zymosterol	Ergosterol	24(28)-Dehydro-ergosterol
Campesterol (4)	91.2	4.7	-	4.1
Cholesterol (5)	90.2	-	8.4	1.3
22,23-Dihydro-brassicasterol (3)	65.4	22.4	-	12.2
Ergosterol (4)	39.1	52.3		8.6
β-Sitosterol (3)	68.0	6.1	25.9	-
Stigmasterol (4)	59.8	12.3	26.0	1.6

Figures in parentheses indicate the number of analyses conducted on independently-grown batches of cells. The 95% confidence limits of the values for the sterols supplemented to the medium were somewhat greater for the esterified sterols than for the free sterols, because of the smaller sizes of these fractions (see Table 5).

TABLE 5

TOTAL LIPID CONTENT AND LIPID COMPOSITION OF *Saccharomyces cerevisiae* N.C.Y.O. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF DIFFERENT STEROLS.

Sterol included in medium	Content (mg/100mg dry wt. cells) of					
	Total lipid	Free sterol	Esterified sterol	Phospholipid	Triacyl-glycerol	Squalene
Campesterol	9.87 ± 0.19 (4)	0.17 ± 0.01 (4)	0.07 ± 0.04 (4)	3.45 ± 0.40 (5)	N D	N.D
Cholesterol	11.25 ± 1.14 (14)	0.16 ± 0.05 (9)	0.21 ± 0.03 (9)	3.69 ± 0.18 (24)	5.72 ± 0.63 (3)	0.13 ± 0.05 (6)
7-Dehydro-cholesterol	8.10 ± 0.83 (6)	0.10 ± 0.02 (6)	0.04 ± 0.02 (6)	3.56 ± 0.13 (10)	N D	N D
Ergosterol	9.52 ± 0.84 (11)	0.13 ± 0.04 (8)	0.02 ± 0.01 (4)	3.49 ± 0.16 (20)	3.68 ± 0.60 (3)	0.18 ± 0.05 (4)
β-Sitosterol	9.34 ± 3.10 (4)	0.13 ± 0.02 (3)	0.03 ± 0.03 (4)	3.19 ± 0.33 (5)	N D	N D
Stigmasterol	9.96 ± 3.12 (3)	0.18 ± 0.02 (4)	0.05 ± 0.05 (4)	3.38 ± 0.13 (4)	N D	N D

Figures in parentheses indicate the number of analyses conducted on independently-grown batches of cells. The values are quoted ± 95% confidence limits. ND indicates that the value was not determined.

in the presence of cholesterol, where the higher content was largely accounted for by an increased content of triacylglycerols. The contents of other classes of lipid are remarkably similar in cells grown in the presence of the different sterols. An interesting exception is with the content of esterified sterol in cells grown in medium containing cholesterol, which is at least three times greater than the content in cells grown in the presence of any of the other cells.

Tween 80 was included in the medium for anaerobic growth of the yeast to supply $C_{18:1}$ acid. The phospholipid fraction from cells grown in the presence of different sterols was enriched to the extent of 58% to 63% with $C_{18:1}$ residues (Table 6). However, the fatty-acyl composition of the neutral lipid fraction (esterified sterols and triacylglycerols) was very different, and contained predominantly $C_{16:0}$, together with some $C_{14:0}$ and $C_{18:0}$ acids (Table 7).

Table 8 summarizes the composition of the saturated and unsaturated fatty-acyl residues from the phospholipids and neutral lipids.

The phospholipid composition of cells grown in the presence of each of the sterols except 22,23-dihydrobrassicasterol (Table 9) showed this to be remarkably constant. The greatest variation was in the contents of phosphatidyl-inositol.

TABLE 6

COMPOSITION OF FATTY-ACYL RESIDUES FROM THE PHOSPHOLIPIDS OF Saccharomyces cerevisiae N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF TWEEN 80 AND DIFFERENT STEROLS

Sterol included in medium	Percentage composition of the fatty-acyl residues from the phospholipids						
	14:0	15:0	16:0	16:1	16:2	18:0	18:1
Campesterol	2.0 ± 0.5	tr.	17.9 ± 2.3	12.8 ± 0.5	1.3 ± 0.2	2.5 ± 0.7	63.4 ± 2.6
Cholesterol	2.2 ± 0.4	1.3 ± 0.3	15.5 ± 3.8	16.7 ± 3.4	-	2.1 ± 2.1	61.9 ± 3.6
7-Dehydro cholesterol	4.2 ± 2.4	1.6 ± 0.9	17.3 ± 21.9	12.2 ± 6.6	1.2 ± 0.6	5.1 ± 6.4	58.2 ± 5.5
22,23-Dihyro-brassicasterol	1.9 ± 1.8	tr.	18.6 ± 0.5	11.9 ± 3.3	1.4 ± 0.1	3.6 ± 1.0	62.4 ± 0.4
Ergosterol	2.9 ± 1.2	1.4 ± 0.7	18.3 ± 2.2	18.9 ± 1.5	-	tr.	58.2 ± 7.7
β-Sitosterol	2.3 ± 0.1	tr.	18.7 ± 2.0	12.2 ± 0.4	1.3 ± 0.3	2.2 ± 0.6	63.2 ± 1.9
Stigmasterol	2.9 ± 0.3	tr.	20.5 ± 3.7	13.0 ± 0.5	1.1 ± 0.3	3.1 ± 0.9	59.3 ± 4.1

Values quoted are the means of three separate analyses ± 95% confidence limits. tr. indicates trace (less than 1% of the total).

TABLE 7

COMPOSITION OF THE FATTY-ACYL RESIDUES FROM THE NEUTRAL LIPIDS OF *Saccharomyces cerevisiae* N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF TWEEN 80 AND DIFFERENT STEROLS

Sterol included in medium	Percentage composition of the fatty-acyl residues from the neutral lipids						
	12:0	14:0	15:0	16:0	16:1	17:0	18:0
Campesterol	2.1 ± 0.6	16.9 ± 1.2	2.1 ± 0.3	58.6 ± 1.4	2.7 ± 0.4	1.6 ± 0.1	15.8 ± 1.4
Cholesterol	3.9 ± 1.9	17.7 ± 2.0	3.4 ± 1.3	56.7 ± 5.8	3.5 ± 3.5	tr.	14.4 ± 1.2
7-Dehydro-cholesterol	4.4 ± 3.2	19.9 ± 26.0	6.5 ± 8.8	43.2 ± 15.5	1.0 ± 2.2	10.4 ± 32.0	14.3 ± 6.4
22,23-Dihydro-brassicasterol	4.1 ± 3.3	17.2 ± 0.6	3.4 ± 0.4	55.2 ± 3.5	tr.	2.7 ± 3.4	17.2 ± 3.7
Ergosterol	4.9 ± 3.2	16.8 ± 8.3	2.8 ± 0.6	57.6 ± 13.3	2.4 ± 1.1	tr.	15.5 ± 4.0
β-Sitosterol	2.7 ± 1.0	18.5 ± 1.1	2.0 ± 1.0	60.0 ± 6.4	3.1 ± 1.1	tr.	13.6 ± 2.3
Stigmasterol	6.4 ± 3.4	22.5 ± 9.9	4.0 ± 1.0	48.7 ± 4.4	2.9 ± 1.4	tr.	15.3 ± 3.8

Values quoted are the means of three separate analyses $\pm_{\lambda}^{95\%}$ confidence limits. tr. indicates trace (less than 1% of the total)

TABLE 8

PERCENTAGE COMPOSITION OF TOTAL SATURATED AND TOTAL UNSATURATED FATTY-ACYL RESIDUES IN PHOSPHOLIPIDS AND NEUTRAL LIPIDS OF *Saccharomyces cerevisiae* N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF TWEEN 80 AND DIFFERENT STEROLS

Sterol included in medium	Percentage composition of saturated fatty-acyl residues in		Percentage composition of unsaturated fatty-acyl residues in	
	Phospholipids	Neutral lipids	Phospholipids	Neutral lipids
Campesterol	22.4	97.1	77.5	2.7
Cholesterol	21.1	96.1	78.6	3.5
7-Dehydro-cholesterol	28.2	98.7	71.6	1.0
22,23-Dihydro-brassicasterol	24.1	99.8	75.7	tr.
Ergosterol	22.6	97.6	77.1	2.4
β-Sitosterol	23.2	96.8	76.7	3.1
Stigmasterol	26.5	96.9	73.4	2.9

TABLE 9

PHOSPHOLIPID COMPOSITION OF *Saccharomyces cerevisiae* N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF DIFFERENT STEROLS

Sterol included in medium	Content of Phospholipid (mg/100mg dry wt. cells)					
	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidic Acid	Lysophospho-lipids
Campesterol (5)	2.03 ± 0.12	0.64 ± 0.17	0.32 ± 0.12	0.22 ± 0.14	0.10 ± 0.09	0.15 ± 0.05
Cholesterol (6)	1.99 ± 0.22	0.68 ± 0.06	0.83 ± 0.46	0.22 ± 0.05	0.11 ± 0.03	0.16 ± 0.09
7-Dehydro-cholesterol (5)	1.74 ± 0.21	0.66 ± 0.08	0.63 ± 0.25	0.26 ± 0.08	0.13 ± 0.02	0.14 ± 0.14
Ergosterol (5)	1.71 ± 0.14	0.62 ± 0.11	0.42 ± 0.20	0.28 ± 0.12	0.13 ± 0.02	0.28 ± 0.16
β-Sitosterol (3)	1.82 ± 0.22	0.51 ± 0.10	0.58 ± 0.21	0.14 ± 0.15	0.03 ± 0.06	0.10 ± 0.12
Stigmasterol (4)	1.55 ± 0.27	0.45 ± 0.12	0.65 ± 0.35	0.15 ± 0.09	0.05 ± 0.01	0.24 ± 0.19

Figures in parentheses indicate the number of analyses conducted on independently-grown batches of cells. The values are quoted ± 95% confidence limits. Trace amounts of cardiolipin (0.02) were detected in cells grown in the presence of cholesterol or ergosterol.

Preparation and analysis of sphaeroplasts

Alterthum and Rose (1973) reported differences in the susceptibility of Sacch. cerevisiae N.C.Y.C. 366, grown anaerobically in the presence of ergosterol and different unsaturated fatty acids, to the action of Basidiomycete glucanase which they attributed to alterations in wall composition caused by changes in the lipid composition of the plasma membrane. Cells enriched with various sterols differed slightly but consistently in their susceptibility to the action of the wall-dissolving glucanase, as judged by the time taken for the absorbance of portions of the glucanase-containing reaction mixture when added to water to decline to a constant low value (Table 10). Populations of cells grown in the presence of ergosterol or stigmasterol took slightly longer to be converted completely into sphaeroplasts compared with cells grown in medium containing one of the other sterols.

During preparation of sphaeroplasts, portions of the glucanase-containing reaction mixture were routinely diluted into buffered 1.2M sorbitol (see Methods). As incubation of the reaction mixture proceeds, there is, with aerobically-grown cells, a drop of not more than 10% in the absorbance of the diluted suspension (Cartledge and Rose, 1973). This pattern of change was observed with cells grown anaerobically in the presence of any of the sterols, with the exception of cholesterol or 7-dehydrocholesterol (Table 10). The absorbance of diluted

TABLE 10

EFFECT OF STEROL COMPOSITION OF *Saccharomyces cerevisiae* N.C.Y.C. 366 ON SUSCEPTIBILITY OF CELLS TO GLUCANASE ACTION AND ON THE STABILITY OF SPHAEROPLASTS

Sterol included in medium	Time (min) taken to complete sphaeroplast formation	Percent ratio of final to original absorbance of sphaeroplasts diluted into glucanase-free buffered sorbitol
Campesterol	20 (4)	84 ± 13 (4)
Cholesterol	15 (7)	73 ± 7 (6)
7-Dehydro-cholesterol	18 (5)	72 ± 16 (5)
22,23-Dihydro-brassicasterol	20 (4)	88 ± 10 (4)
Ergosterol	24 (3)	91 ± 10 (4)
β-Sitosterol	21 (5)	92 ± 6 (5)
Stigmasterol	27 (5)	94 ± 4 (5)

Sphaeroplast formation was carried out as described previously (Alterthum and Rose, 1973; Cartledge and Rose, 1973), and was monitored by diluting portions (0.1ml) of the suspension in glucanase-containing buffered sorbitol (1.2M) into 2.9ml portions of water, gently shaking the diluted suspension and measuring the absorbance (E^{600}_{1cm}). Values in the right hand column were obtained by diluting portions (0.1ml) of the reaction mixture into 2.9ml portions of buffered sorbitol and measuring the absorbance (E^{600}_{1cm}). Figures in parentheses indicate the numbers of tests conducted on independently-grown batches of cells. The values in the right hand column are quoted ± 95% confidence limits.

suspensions from reaction mixtures containing cells grown in the presence of either of these sterols declined to almost 70% of the value at zero time. There appears to be two possible explanations for this increased drop in absorbance. One possibility was that sphaeroplasts with plasma membranes enriched in cholesterol or 7-dehydrocholesterol assume a volume in 1.2M sorbitol different from that taken up by sphaeroplasts enriched in any one of the other sterols. A second possibility was that, with sphaeroplasts enriched with cholesterol or 7-dehydrocholesterol, there was sufficient lysis in 1.2M sorbitol to cause an appreciable decrease in absorbance. Microscopic examination showed that there was only a small amount of lysis of these sphaeroplasts in 1.2M sorbitol.

Although chloramphenicol and cycloheximide were included in reaction mixtures and buffers during preparation of sphaeroplasts, it was possible that some endogenous synthesis of sterols took place during sphaeroplast formation. To examine this possibility, freshly prepared washed sphaeroplasts were freeze dried, and their free-sterol content analysed. These analyses revealed that the percentage enrichment of the free-sterol fraction with the exogenously supplied sterol either remained unchanged or decreased by no more than 10% of the total (Table 11). Exceptions to this generalisation were noted with sphaeroplasts enriched with β-sitosterol and campesterol where the percentage enrichments fell by, respectively, 23 and 15% of the total free sterols in whole-cell lipids (see

TABLE 11

COMPOSITION OF THE FREE STEROLS OF SPHAEROPLASTS PREPARED FROM Saccharomyces cerevisiae N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF DIFFERENT STEROLS.

Sterol included in medium	Percentage composition of the free sterols			
	Sterol included in medium	Zymosterol	Ergosterol	24(28)-Dehydro -Ergosterol
Campesterol	77.0	14.1	-	8.9
Cholesterol	81.8	-	8.6	9.2
22,23-Dihydro-brassicasterol	77.6	22.4	-	-
β-Sitosterol	60.3	14.2	25.5	-
Stigmasterol	81.2	11.0	-	7.8

Table 3). The changes were due to the appearance in sphaeroplasts of sterols that are synthesized by aerobically grown cells.

Preparation and analysis of isolated plasma membranes

Plasma membranes from sphaeroplasts of Sacch. cerevisiae N.C.Y.C. 366, enriched in cholesterol or stigmasterol, were radioactively labelled with ^{125}I, washed and separated on a discontinuous sucrose density gradient.

When 3ml aliquots of lysed sphaeroplasts were applied to a gradient consisting of solutions containing 5ml each of 10, 20, 30, 40, 50 and 60% (w/v) sucrose large concentrations of radioactivity were found throughout the gradient including the pellet at the bottom of the tube (Fig. 4). Much of this activity was associated with a fraction floating on the surface of the gradient which previous work in this laboratory has shown to consist of large and small lipid-containing vesicles (Hossack et al., 1973). However when the sphaeroplast lysates were themselves centrifuged at 3,000g for 10 min, and the membrane-containing pellet washed, then 80% of the counts associated with the cholesterol-enriched sphaeroplasts and 70% from those enriched with stigmasterol were removed with the washings. When these membrane-containing pellets were applied to the gradient described in Methods the highest concentration of radioactivity was found in the pellet at the bottom of the

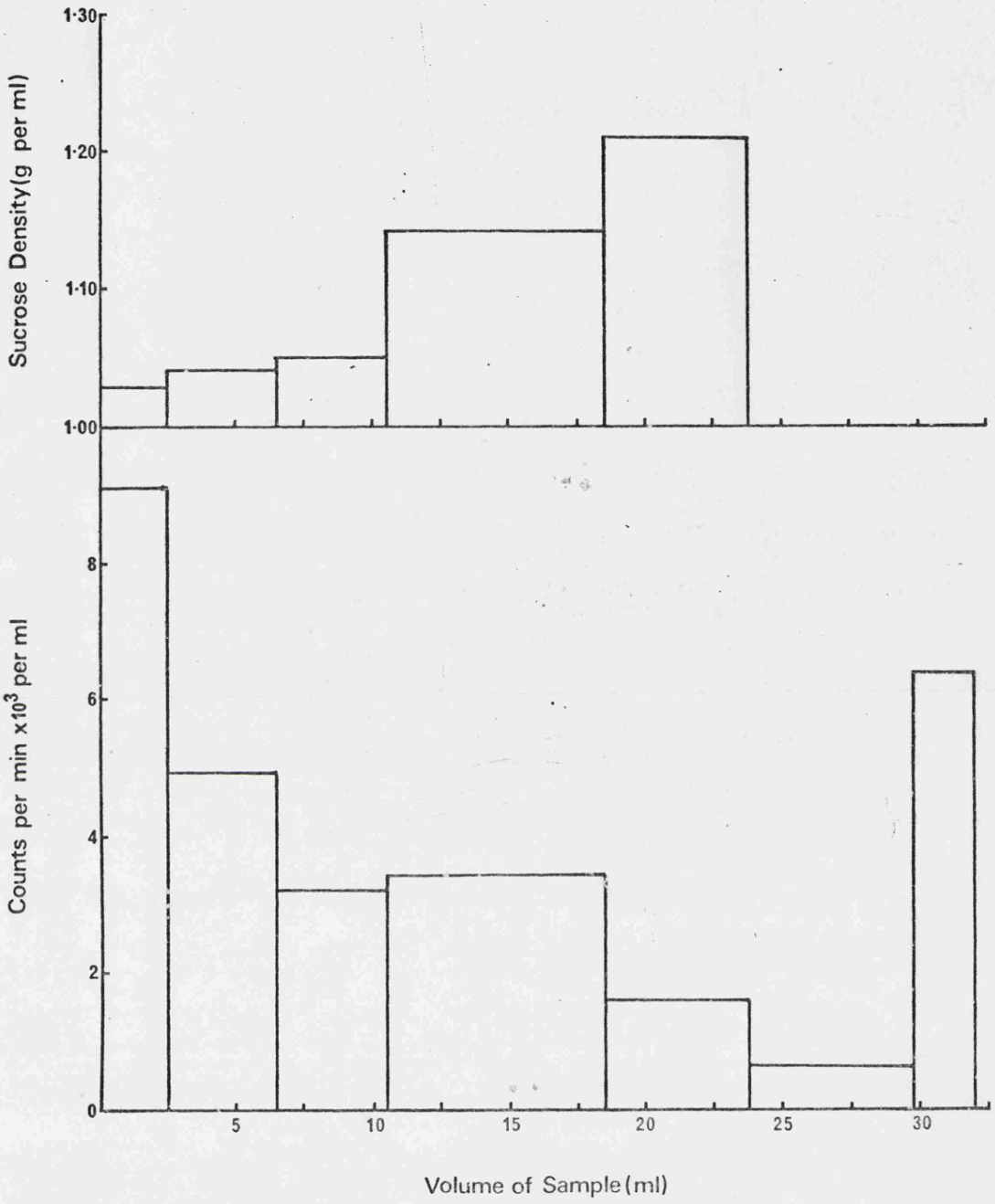


Fig. 4 Histograms showing density and distribution of radioactivities on a sucrose density gradient of a lysed preparation of labelled sphaeroplasts of Saccharomyces cerevisiae N.C.Y.C. 366 enriched in cholesterol. These show (a) sucrose density (g/ml) and (b) counts per minute per ml of sample.

gradient, with a density of just above 1.30g/ml (Figs. 5 and 6, Table 12). The location of radioactivity on the gradient approximately paralleled the absorbance at 280nm (Figs. 5 and 6).

After sucrose density gradient centrifugation the pellets were freeze dried and the free sterol analyses were carried out. These showed that about 70% of the free sterol of the pellet was the one included in the medium (Table 13).

Fragility of plasma membranes

The fragility of the plasma membrane surrounding the sphaeroplast was assessed by examining the stability of sphaeroplasts when suspended in hypotonic solutions of buffered sorbitol. When sphaeroplasts were suspended in buffer containing sorbitol at 1.0M or less, there was a decrease in the absorbance of the suspension. When suspended in buffer containing 0.9 - 0.7M sorbitol, two different patterns of responses were detected (Fig. 7, Table 14). Sphaeroplasts enriched in ergosterol or stigmasterol were comparatively stable as judged by the small decline in the absorbance of the suspension and microscopically by the absence of ruptured sphaeroplasts. When sphaeroplasts enriched in campesterol, cholesterol, 7-dehydrocholesterol, 22,23-dihydrobrassicasterol or β-sitosterol, there was a much larger decline in absorbance.

Fig. 5. Histograms showing density, distribution of radioactivity and absorbance at 280nm on a sucrose density gradient of a membrane preparation obtained from radio-actively labelled sphaeroplasts of Saccharomyces cerevisiae N.C.Y.C. 366 enriched in cholesterol.

These show (a) sucrose density (g/ml), (b) counts per minute per ml of sample and (c) absorbance (E^{280}_{1cm})

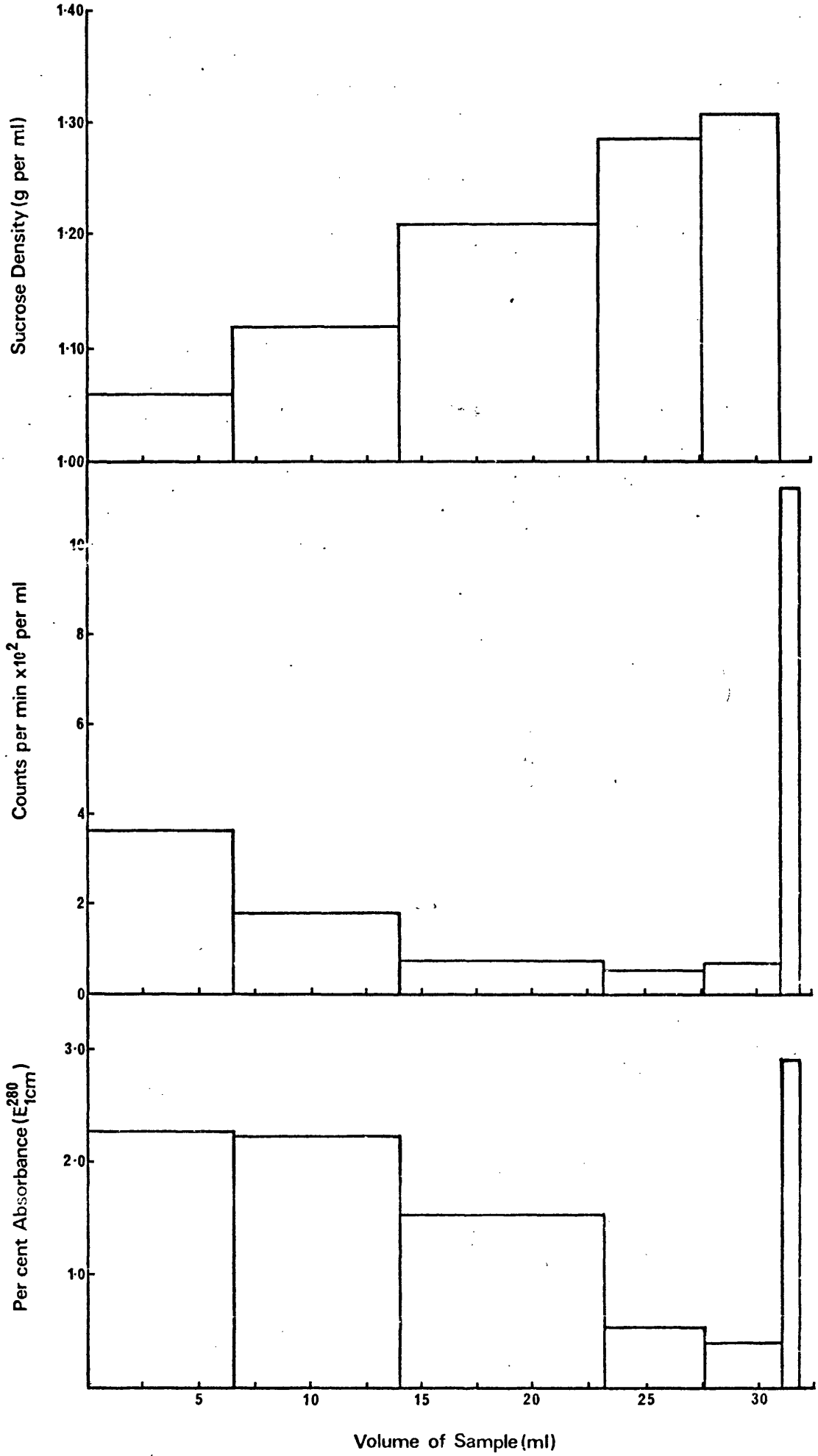

Sucrose Density (g per ml)
1·40
1·30
1·20
1·10
1·00
Counts per min x10² per ml
8
6
4
2
0
Per cent Absorbance (E^{280}_{1cm})
3·0
2·0
1·0
5
10
15
20
25
30
Volume of Sample (ml)

Fig. 6. Histograms showing density, distribution of radioactivity and absorbance at 280nm on a sucrose density gradient of a membrane preparation obtained from radio-actively labelled sphaeroplasts of Saccharomyces cerevisiae N.C.Y.C. 366 enriched in stigmasterol.

These show (a) sucrose density (g/ml),
(b) counts per minute per ml of sample and
(c) absorbance (E^{280}_{1cm})

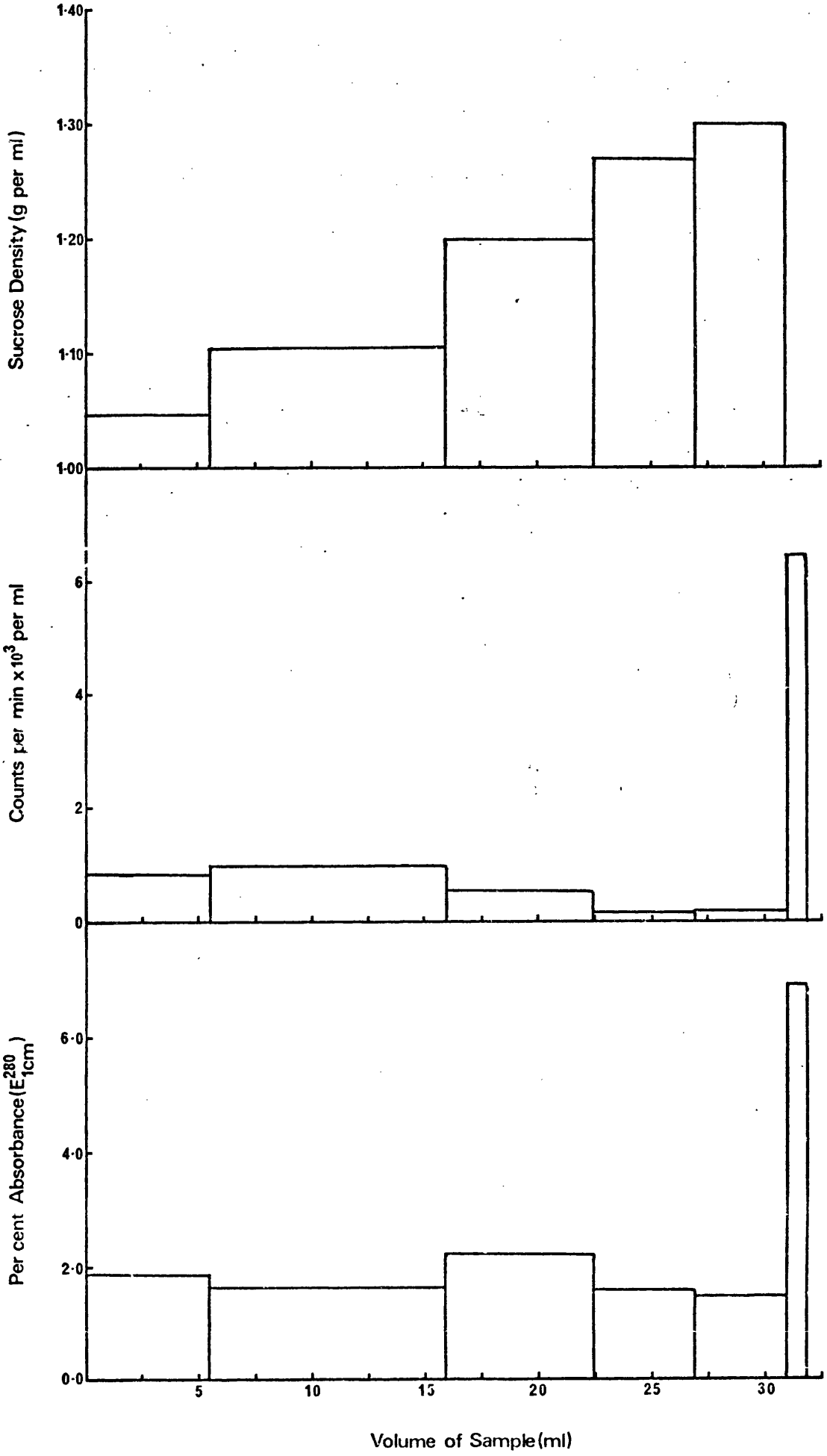

Sucrose Density (g per ml)
1·40
1·30
1·20
1·10
1·00
Counts per min x 10³ per ml
6
4
2
0
Per cent Absorbance (E 280 1cm)
6·0
4·0
2·0
0·0
5
10
15
20
25
30
Volume of Sample (ml)

TABLE 12

DISTRIBUTION OF RADIOACTIVITY FROM SPHAEROPLASTS PREPARED FROM *Saccharomyces cerevisiae* N.C.Y.C. 366 BETWEEN THE 3000g MEMBRANE-CONTAINING PELLET AND WASHINGS

Sterol included in medium	Percentage radioactivity from		
	Total sphaeroplast lysate	Washings from 3000g membrane-containing pellet	3000g membrane-containing pellet
Cholesterol	100	79.5	20.5
Stigmasterol	-	69.5	30.5

TABLE 13

COMPOSITION OF THE FREE STEROLS OF PLASMA MEMBRANES OBTAINED FROM Saccharomyces cerevisiae N.C.Y.C. 366 GROWN ANAEROBICALLY IN THE PRESENCE OF CHOLESTEROL OR STIGMASTEROL

Sterol included in medium	Percentage composition of the free sterols			
	Sterol included in medium	Zymosterol	Ergosterol	24(28)-Dehydro-ergosterol
Cholesterol	71.3	-	3.7	25.0
Stigmasterol	70.1	19.7	2.3	7.7

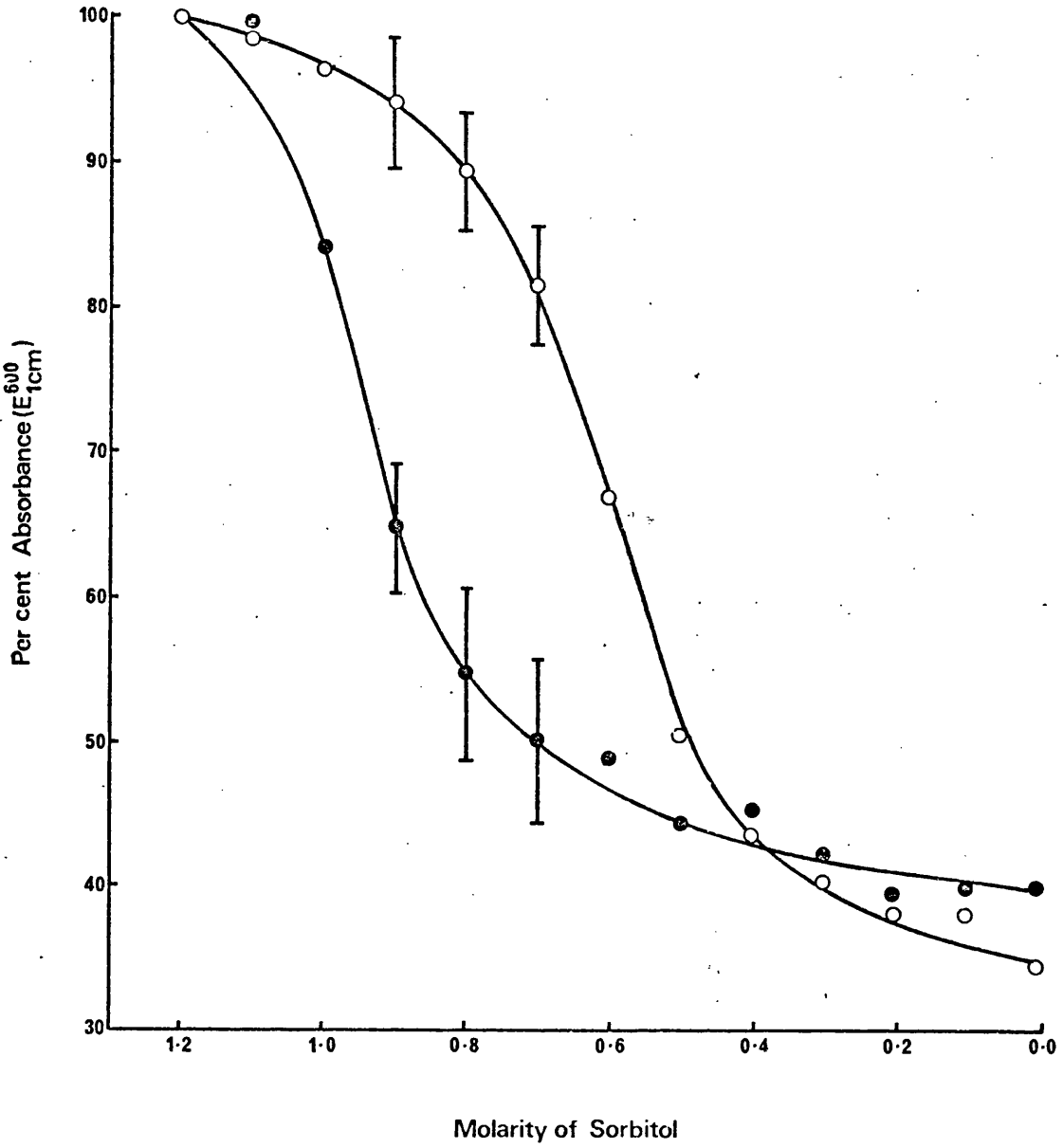


Fig. 7. Stability of sphaeroplasts from cells enriched in cholesterol (●) or ergosterol (○) when suspended in hypotonic solutions of buffered sorbitol. The vertical bars indicate 95% confidence limits of values.

TABLE 14

STABILITY OF SPHAEROPLASTS OF *Saccharomyces cerevisiae* N.C.Y.C. 366 ENRICHED IN DIFFERENT STEROLS AFTER DILUTION INTO HYPOTONIC SOLUTIONS OF BUFFERED SORBITOL

Sterol included in medium	Per cent ratio of absorbance of hypotonic to isotonic suspensions of sphaeroplasts in buffered sorbitol at		
	0.9M	0.8M	0.7M
Campesterol (4)	67 ± 6.1	60 ± 5.0	55 ± 5.8
Cholesterol (4)	65 ± 10.9	55 ± 7.0	50 ± 4.2
7-Dehydro-cholesterol (5)	70 ± 11.4	66 ± 15.0	64 ± 9.7
22,23-Dihydro-brassicasterol (4)	67 ± 9.7	62 ± 5.6	61 ± 10.6
Ergosterol (3)	94 ± 9.4	89 ± 7.4	82 ± 8.0
β-Sitosterol (5)	77 ± 9.7	68 ± 7.2	60 ± 5.8
Stigmasterol (5)	96 ± 3.9	93 ± 4.1	87 ± 8.2

Figures in parentheses indicate the number of tests conducted on independently-prepared batches of sphaeroplasts. The values are quoted ± 95% confidence limits.

The effects caused by suspending sphaeroplasts in hypotonic solutions of buffered sorbitol were further examined by measuring the volume distribution of surviving sphaeroplasts in the suspensions by electronic sizing. The data (Figure 8) show that the average volume of surviving sphaeroplasts increased as the sorbitol concentration was lowered, slightly more so with sphaeroplasts enriched in cholesterol than with ergosterol. It was established (Victoria Sharpe, unpublished observations) that the effects of sorbitol and sodium chloride concentrations are additive as far as osmotic effects on sphaeroplasts are concerned. Table 15 shows the percentages of sphaeroplasts that survive in the hypotonic solutions of buffered sorbitol. It can be seen that sphaeroplasts enriched in ergosterol were more stable when diluted into buffer containing 0,8 or 0.6M sorbitol than sphaeroplasts enriched in cholesterol.

Force area studies on phospholipids and sterols

Mixtures of phosphatidylcholine and either cholesterol or ergosterol at different molar ratios were applied as a monolayer onto the surface of a Langmuir trough. From the force-area curves so obtained the mean molecular areas of the mixtures of lipids were found at a pressure of 5 dynes/cm. These areas were somewhat less than those expected if there was no interaction between the phospholipid and the sterol. The extent of this 'condensing'

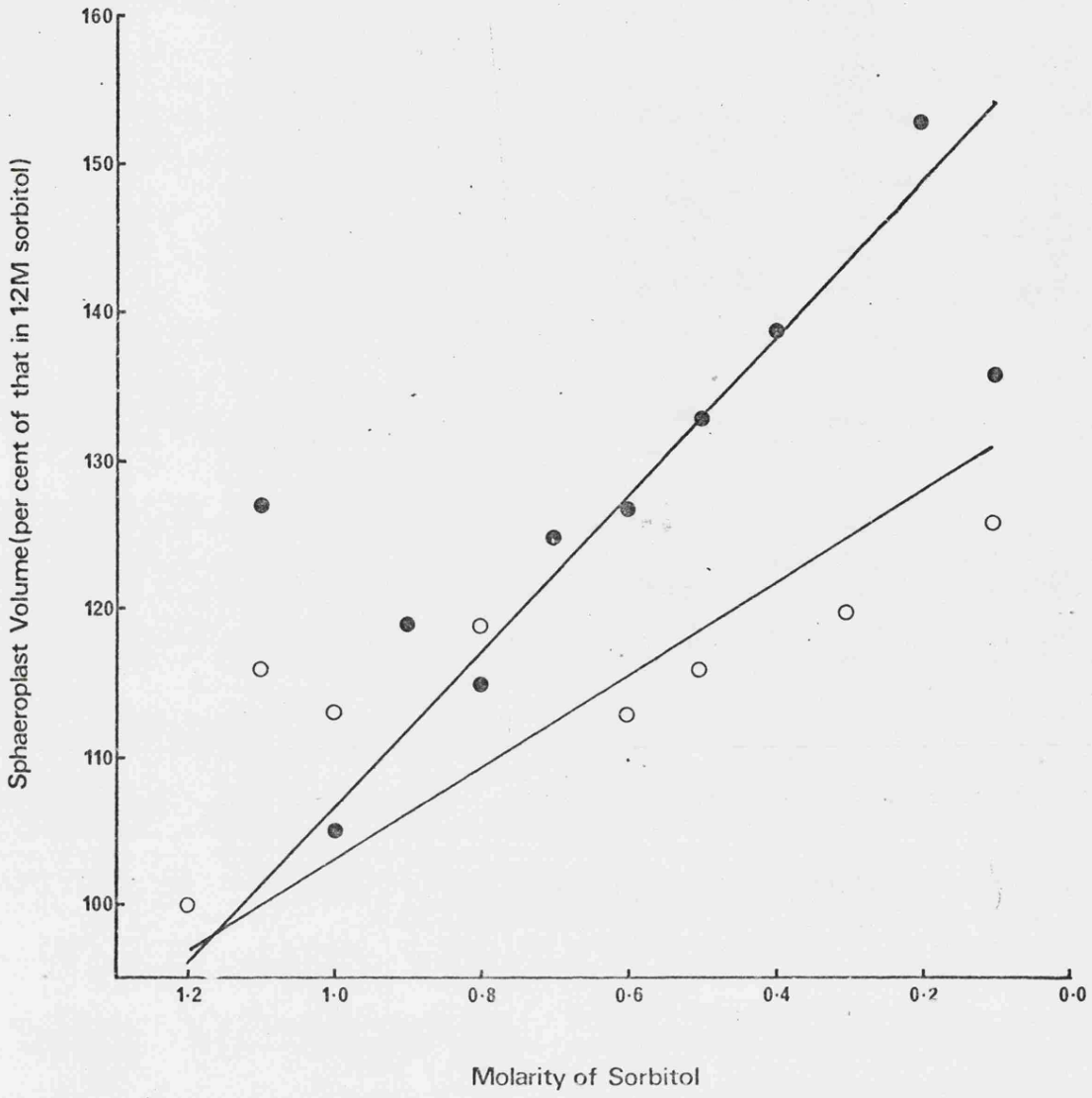


Fig. 8. Percentage increase in average volumes of surviving sphaeroplasts enriched in cholesterol (●) or ergosterol (○) after dilution into hypotonic solutions of buffered sorbitol. The lines of best fit were calculated by regression analysis.

TABLE 15

SURVIVAL OF SPHAEROPLASTS ENRICHED IN CHOLESTEROL OR ERGOSTEROL AFTER DILUTION INTO HYPOTONIC SOLUTIONS OF BUFFERED SORBITOL

Molarity of sorbitol in buffer	Survival of sphaeroplasts enriched in Cholesterol		Ergosterol	
	Number of sphaeroplasts per ml x 10^6	Per cent of number in buffered 1.2M sorbitol	Number of sphaeroplasts per ml x 10^6	Per cent of number in buffered 1.2M sorbitol
1.2	5.55	100	4.28	100
0.8	2.45	44.1	3.35	78.3
0.6	1.76	31.7	1.84	43.3

Sphaeroplasts were formed and diluted into buffered sorbitol as described in Methods. The numbers of surviving sphaeroplasts in the dilutions were measured after 10 min, using a haemocytometer microscope slide.

effect was similar for each type of sterol (Figs. 9 and 10) and the maximum effect occured at a phosphatidyl-choline : sterol molar ratio of approximately 2 : 1.

Similar studies on phosphatidyl-myo-inositol and cholesterol or ergosterol showed a maximum 'condensing' effect at a molar ratio of approximately 1 : 1 (Figs. 11 and 12). However above a mole fraction of 65% phosphatidyl-myo-inositol with cholesterol there was an apparent repulsion between the lipids, which was not observed in the case of ergosterol.

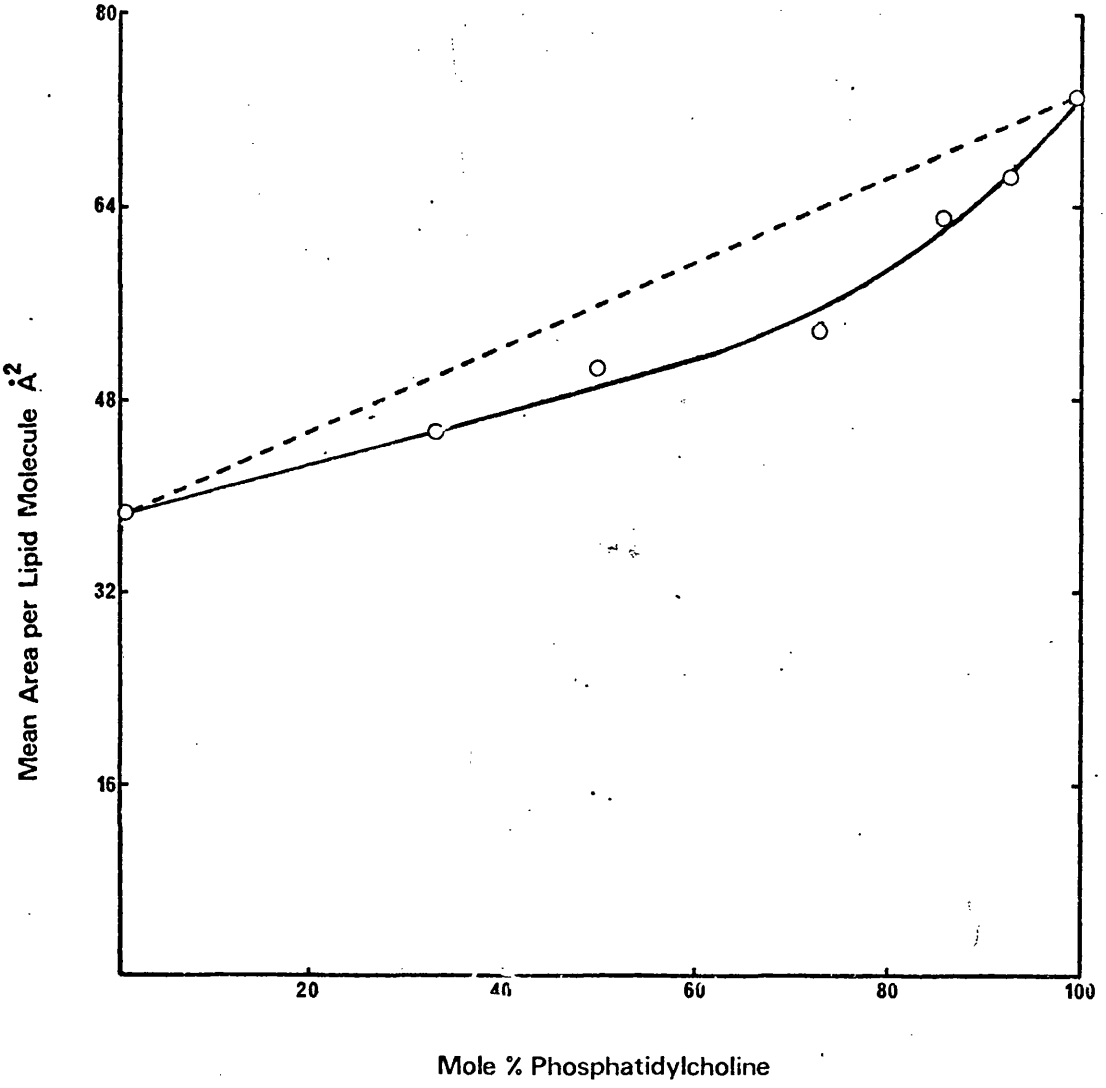


Fig. 9. Variation of the mean area per lipid molecule, as a function of composition, of mixed monolayers of phosphatidylcholine and cholesterol at a pressure of 5 dynes per cm.

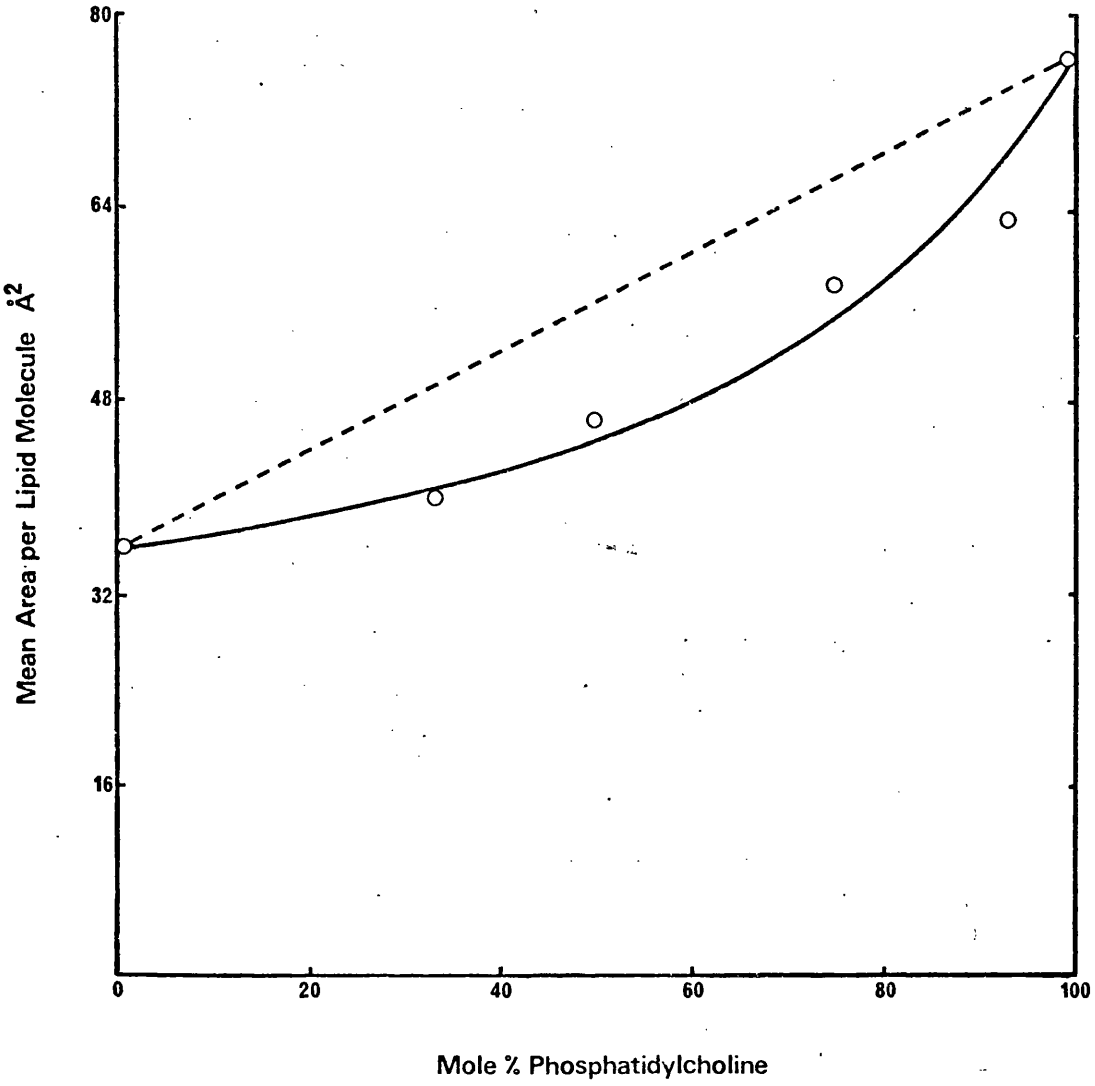


Fig. 10. Variation of the mean area per lipid molecule, as a function of composition, of mixed monolayers of phosphatidylcholine and ergosterol at a pressure of 5 dynes per cm.

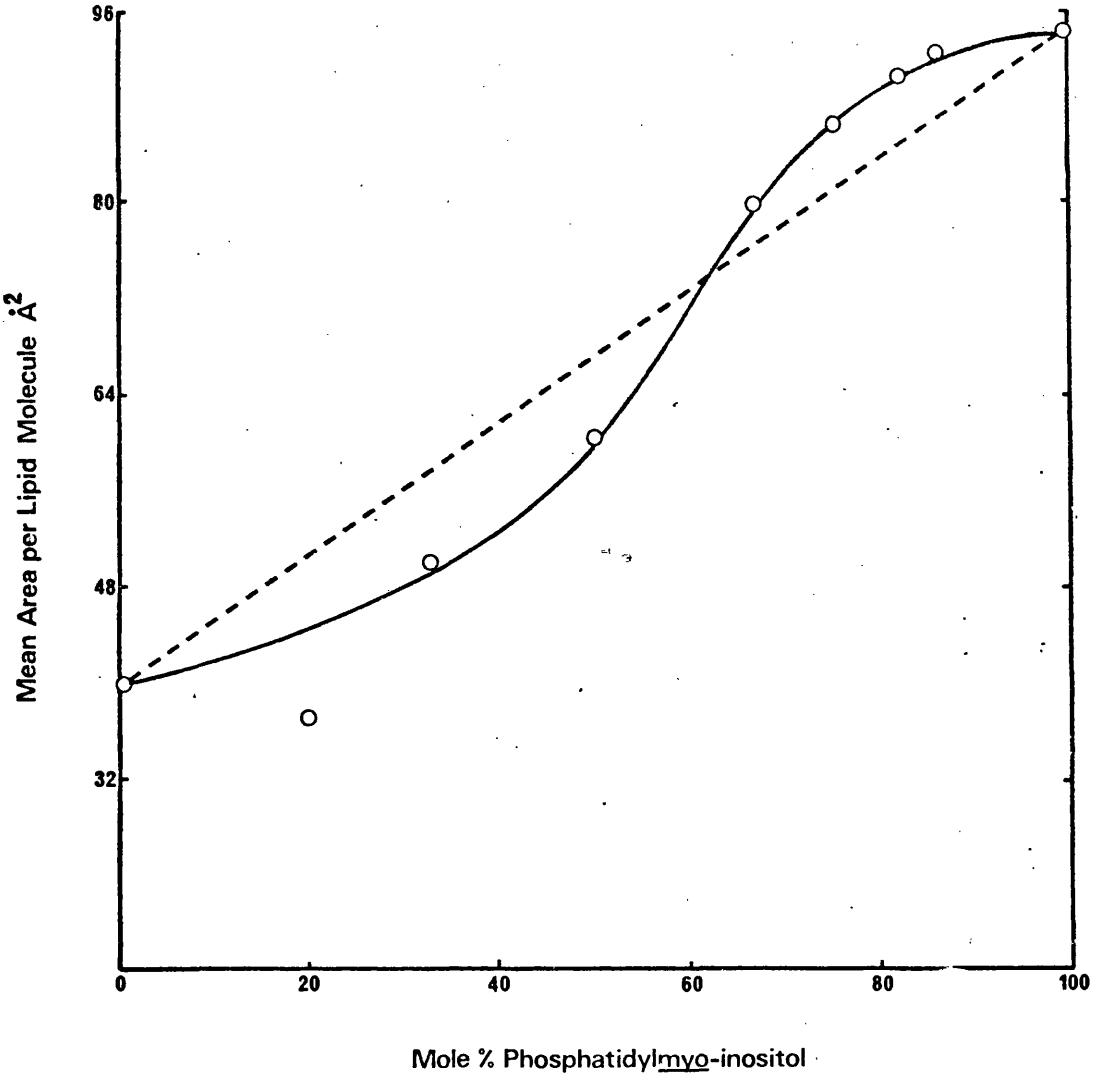


Fig. 11. Variation of the mean area per lipid molecule, as a function of composition, of mixed monolayers of phosphatidyl-myo-inositol and cholesterol at a pressure of 5 dynes per cm.

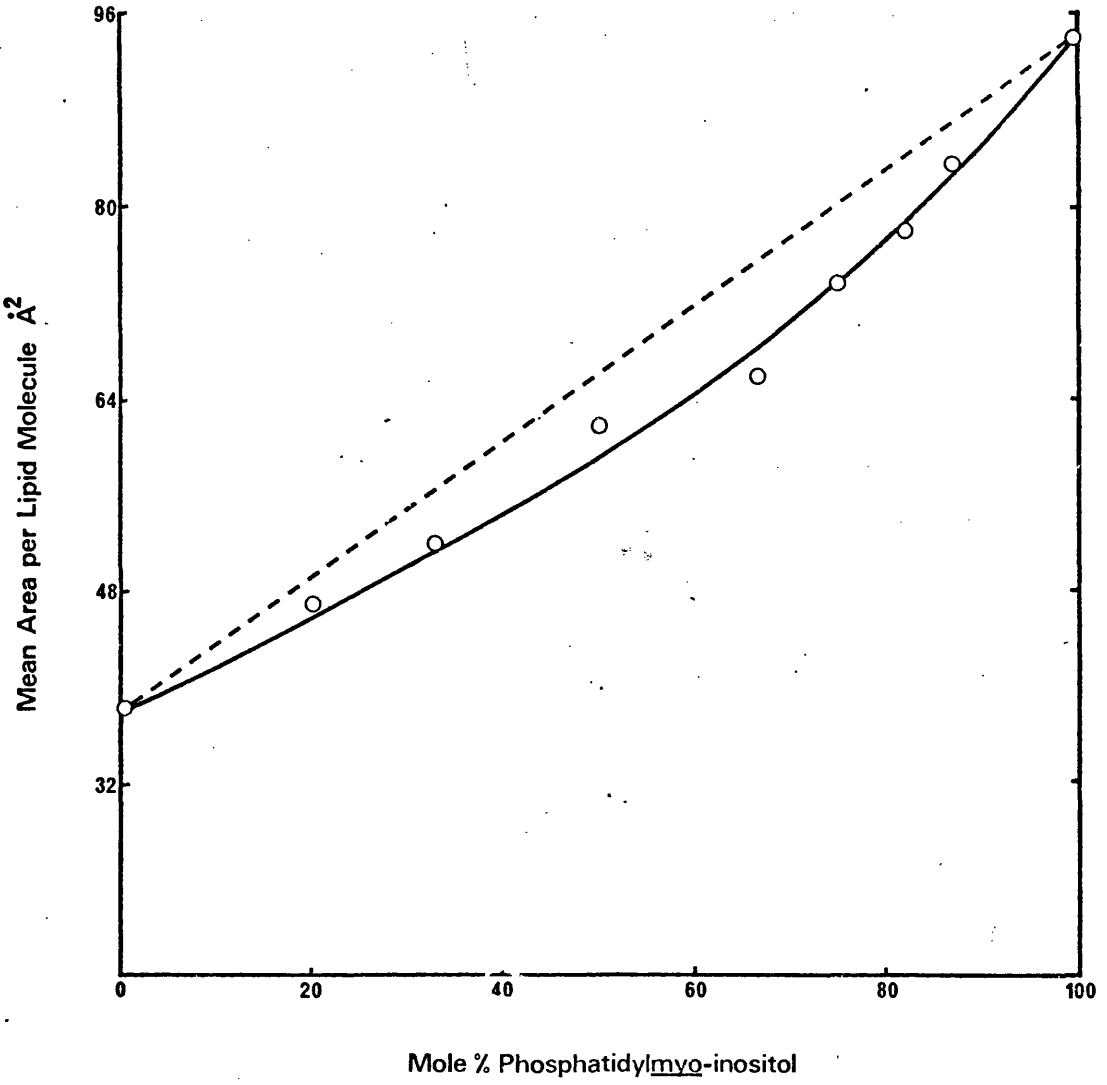


Fig. 12. Variation of the mean area per lipid molecule, as a function of composition, of mixed monolayers of phosphatidyl-myo-inositol and ergosterol at a pressure of 5 dynes per cm.

DISCUSSION

The data reported in this thesis have been obtained by exploiting the anaerobic requirement by Sacch. cerevisiae for a sterol and an unsaturated fatty acid (Andreasen and Stier, 1954) and they demonstrate the advantages of using this system. The study was restricted to varying the sterol composition of the yeast membranes, Tween 80 being used as a source of oleic acid.

Although sterol-requiring (Karst and Lacroute, 1973; Gollub et al., 1974) and fatty acid-requiring (Resnick and Mortimer, 1966) mutants of yeast can also be obtained enriched with a particular sterol or fatty-acyl residue they have not yet been exploited particularly with regard to studies on the role of sterols in the plasma membrane. Lack of physiological work on these mutants may be due to their genetic instability and also because they are often auxotrophic for other nutrients such as haem.

Lipid Composition of Anaerobically grown cells

Before discussing the physical properties of sphaeroplasts of Sacch. cerevisiae N.C.Y.C. 366 in response to altering the sterol composition, I wish to discuss the effects of sterol enrichments on the lipid content and composition of the cells. It would appear that the sterols added to the growth medium are incorporated into membranes in chemically unmodified form. The degree of incorporation was high,

especially of free sterols. This was particularly important as free sterols are one of the major lipids present in cell membranes. It was also noted that the rates of growth of cells enriched with any one of the sterols were similar, and that cells enriched with cholesterol or ergosterol were of similar size. These findings suggest that the chemical nature of the sterol in membranes does not materially affect the overall regulation of growth rate and cell size, which is an important consideration when the object of the study was to alter a specific component (namely membrane sterol) in the organism. The other sterols detected in anaerobically grown cells, namely zymosterol, ergosterol and 24(28)-dehydroergosterol, probably arise from endogenous biosynthesis *via* squalene, due to the presence of very small amounts of oxygen in the purified nitrogen. Calculations show that the endogenous sterols present in the cells in the inoculum could account for only about 1% of the total free sterol in the harvested crop. The contents of free sterols in anaerobically-grown cells were found to be about 50% of those found in aerobically grown-cells of the same strain (Hunter and Rose, 1972). In cells grown anaerobically in the presence of sterol and Tween 80, the intracellular membrane system is not so well developed as true mitochondria are absent (Morpurgo *et al.*, 1964; Jollow *et al.*, 1968): it is hardly surprizing therefore, that less free sterol was present in these cells.

The total phospholipid contents and phospholipid

compositions of anaerobically-grown cells enriched with any one of the sterols were similar. This is of particular significance, as these lipids, along with sterols, are the principal lipids of cell membranes. The greatest variation, that of the phosphatidylinositol content, is possibly due to the difficulties encountered in extracting this phospholipid as it is more polar than other phospholipids, and may resist extraction by organic solvents. These findings would suggest that the structure of the sterol molecule in a membrane is not concerned with regulation of phospholipid synthesis. This is in agreement with the idea that sterols are not involved in metabolic activities of Sacch. cerevisiae but rather act in a structural role in membranes (Proudlock et al., 1968). The phospholipid compositions of all types of anaerobically-grown cells were similar to those reported by Jollow et al. (1968) on cells of another strain of Sacch. cerevisiae grown in the presence of ergosterol and Tween 80, but contrasted with those obtained by Hunter and Rose (1972) for aerobically-grown Sacch. cerevisiae NCYC 366. The anaerobically-grown cells are deficient in cardiolipin, which is presumably lacking because of the absence of true mitochondria. Also the phosphatidylcholine content of anaerobically-grown cells was found to be 50% higher than in aerobically-grown cells of the same strain (Hunter and Rose, 1972). Perhaps this change in phospholipid composition is due to a demand by the anaerobically-grown cells for a larger proportion of phosphatidylcholine in order to stabilize the cell membranes in the presence of

lower molar proportions of sterol. Alternatively it may be a response by the cells resulting from an enhanced activity of enzymes that catalyse methylation of phosphatidylethanolamine. Perhaps the enhanced activity is due to the lower sterol content, although this would indicate a metabolic role for sterols in the yeast plasma membrane.

The total lipid contents of anaerobically-grown cells enriched with any one of the sterols were lower than in aerobically-grown cells of this strain (Hunter and Rose, 1972). The lower values for the anaerobically-grown cells may be due to smaller amounts of neutral lipid, particularly the triacylglycerols, as was noted by Jollow et al. (1968). However the triacylglycerol values reported by Hunter and Rose (1972) were for aerobically-grown cells lower than those obtained in the present study. The total of the contents of all of the individual lipids reported by these workers accounted for only 56% of the total lipid extracted, whereas in the present study the total values for individual lipid classes obtained from cholesterol- and ergosterol-enriched cells were 88% and 79%, respectively of the total lipid extracted. The lower results obtained by Hunter and Rose (1972) may be due to the different method used for acylglycerol assays.

The total esterified sterol contents of anaerobically-grown cells enriched with any of the sterols were much lower than values reported by Hunter and Rose (1972) using aerobically-

grown cells of the same strain. This is presumably because under anaerobic conditions, there is a smaller pool of intracellular sterol available for esterification since most of the free sterol is incorporated into the plasma membrane as the free alcohol, supplied exogenously. Cholesterol-enriched cells were found to have a somewhat higher esterified sterol content compared with other anaerobically-grown cells, and this might be explained by cholesterol being a more suitable substrate than the other sterols for enzymes that catalyse esterification. The greater proportions of endogenously-synthesized sterol in the esterified form compared to the free alcohols in anaerobically-grown cells enriched in any one of the sterols, is of considerable interest. It may be accounted for in part by endogenously-synthesized sterols present in the starter inoculum, but calculations show this would account for up to only 20% of the esterified sterols in ergosterol-enriched cells and less than 5% in campesterol- or cholesterol-enriched cells which have a higher esterified sterol content than other anaerobically-grown cells. Alternatively, if esterification occurs via a reaction catalysed by a phospholipid-sterol acyltransferase then, following incorporation of exogenously-provided sterol into the plasma membrane, there would remain an intracellular pool of sterol available for esterification made up of a high proportion of endogenously-synthesized sterols.

Squalene analyses of cells grown in the presence of cholesterol or ergosterol not surprisingly showed higher

values than Sacch. cerevisiae grown aerobically (Illingworth et al., 1973). This sterol precursor accumulates under anaerobic conditions since its conversion to sterols requires the presence of molecular oxygen (Dean et al., 1967).

Interesting results were obtained from analyses of fatty-acyl residues from the phospholipids and the neutral lipids of anaerobically-grown cells enriched in any one of the sterols. The major fatty-acyl residues present in the phospholipids were $C_{18:1}$ and this and other unsaturated residues accounted for some 75% of the total, a value comparable with cells of the same strain grown anaerobically in the presence of ergosterol and pure oleic acid (Alterthum and Rose, 1973) or aerobically (Hunter and Rose, 1972). However the fatty-acyl residues of the neutral lipids contained very little unsaturation. These results may give clues to the function of lipid-containing vesicles since, in Sacch. cerevisiae, neutral lipids (sterol esters and triacylglycerols) are thought to be located in low-density intracellular vesicles (Cartledge and Rose, 1973; Hossack et al., 1973) In aerobically- grown cells microsomal acetyl CoA synthetase and fatty-acyl CoA synthetase catalyse synthesis of long-chain fatty-acyl CoA esters (Lynen, 1967). These fatty-acyl residues may be saturated or unsaturated and are incorporated into phospholipids which in turn become membrane constituents. This latter process may occur after transport of the fatty-acyl residues to the membrane, perhaps combined to a lipid

carrier molecule (Sumper and Träuble, 1973) or conceivably in the form of the low density vesicles (Cartledge and Rose, 1973; Hossack et al., 1973; Clausen et al., 1974). It is also possible that, in aerobically-grown yeast, there is synthesis mainly of saturated fatty-acyl chains, which are desaturated when in phospholipids in the membrane (Pugh and Kates, 1973; Talamo et al., 1973). In anaerobically-grown cells oleyl-CoA esters derived from Tween 80, may be incorporated into phospholipids at the site of the growing membrane, while on the other hand microsomal production of fatty-acyl residues via fatty acid synthetase produces only saturated residues, which become incorporated into vesicles before being transferred to the growing membrane.

From the lipid analyses carried out it was found that incorporation of the different sterols did not significantly alter the compositions of other lipids, and so the anaerobic technique was considered to be suitable for studying the effects of making stoicheiometric alteration of the sterol composition of cell membranes, a necessary prerequisite for using these cells in composition-function studies on the plasma membrane.

Formation and Sterol analysis of Sphaeroplasts and Plasma Membranes

The course of sphaeroplast formation was compared using cells

grown anaerobically in the presence of each of the sterols. Although the rates of formation did not vary as dramatically, in contrast to the findings of Alterthum and Rose (1973), who used cells of the same strain grown in the presence of ergosterol and different unsaturated C_{18} acids, it did appear that cells enriched with sterols possessing a double bond at C-22 (ergosterol and stigmasterol) were slightly more resistant to the glucanase-catalysed digestion of cell walls, compared with cells enriched with the other sterols. Sterols have not as yet been shown to be involved in wall-synthesizing processes. However Kotyk (1972) reported that ergosterol, together with a lipoprotein, phosphatidylethanolamine and phosphatidylcholine, is associated with a glucose-binding protein isolated from plasma membranes of Sacch. cerevisiae. These data suggest a role for ergosterol in glucose transport. With anaerobically-grown cells perhaps the presence of ergosterol and stigmasterol in the plasma membrane could cause greater synthesis of cell-wall glucan than does the presence of other sterols. Alternatively these sterols may influence the degree of branching of the cell-wall glucan, and hence its susceptibility to digestion by β-glucanase.

Those cells which were enriched with a sterol possessing an alkyl group at C-24 (campesterol, 22-23 dihydrobrassicasterol, ergosterol, β-sitosterol and stigmasterol) gave rise to sphaeroplasts which were more stable in buffered 1.2M sorbitol (pH 6.0) than those enriched with cholesterol

or 7-dehydrocholesterol. This could be due to the effect of the alkyl group in stabilizing the plasma membrane allowing closer packing of the phospholipid molecules in the membrane. These data should be compared to those of Alterthum and Rose (1973) who, from the same strain of yeast, prepared sphaeroplasts enriched with different fatty-acyl residues, and required the presence of spermine as a stabilizer in suspensions of sphaeroplasts enriched with linolenic acid.

Analysis of the free sterols in sphaeroplasts were carried out in order to check if there was any significant change in the sterol composition after harvesting the cells and their subsequent exposure to molecular oxygen. Throughout the work on anaerobically-grown cells every effort was made to ensure that endogenous synthesis of sterols (and fatty-acyl residues) was minimized. Firstly, the antibiotics actidione and chloramphenicol (Kerridge, 1958; Wilkie, 1970) were included in the buffers during sphaeroplast formation to prevent protein synthesis. Secondly, during harvesting by centrifugation, the temperature was kept low in order to minimize the activities of enzymes which catalyse synthesis of sterols. Thirdly, glassware used during work with sphaeroplasts was flushed with nitrogen gas. Despite these precautions, sterol analyses of sphaeroplasts showed that the degree of enrichment was some 10% lower than that in freeze-dried whole cells, while the degree of enrichment in isolated plasma membranes was about 15% lower than in lipids from

freeze-dried cells. This latter result may have been due to endogenous synthesis, but is more likely due to preferential incorporation of exogenously-supplied sterols in membranes other than plasma membranes.

Membranes were isolated from sphaeroplasts enriched with either cholesterol or stigmasterol, these being representative of each group of osmotically-sensitive sphaeroplasts described in the Results Section. The radio-activity throughout the gradient used to isolate plasma membranes from membrane preparations was low, except in the pellet at the bottom. These findings agree with those reported by Shibeci et al. (1973). However in preliminary experiments on fractionation of sphaeroplast lysates from cholesterol-enriched cells, it was found that considerable radioactivity was located in a fraction which rises to the top of the gradient and which contains low-density lipid-containing vesicles (Hossack et al., 1973). This was not reported by Shibeci et al. (1973), who discarded the supernatant after separating the membrane fraction by centrifugation of lysed sphaeroplasts. One explanation for this finding is that $Na^{125}I$ entered the plasma membrane and labelled the vesicles, which were transiently present in the surface layers of the membrane. This suggestion implies that these vesicles are concerned with synthesis of plasma membranes (Cartledge and Rose, 1973; Hossack et al., 1973; Clausen et al., 1974) or possibly also of cell walls (Sentandreu and Northcote, 1969), as radioactive labelling can only presumably occur when the

vesicles are in close proximity to the outside of the plasma membrane.

Fragility of Sphaeroplast Plasma Membranes

A major finding in the work described in this thesis came from an examination of the stability of sphaeroplasts, derived from anaerobically-grown Sacch. cerevisiae enriched with different sterols, when they were suspended in solutions of buffered sorbitol (pH 6.5). All types of sterol-enriched sphaeroplasts were stable in 1.2M sorbitol but, when these sphaeroplasts were suspended in solutions whose molarities were lower than 1.2M, they became less resistant to osmotic lysis. Two types of response were manifested. Firstly sphaeroplasts enriched in either ergosterol or stigmasterol were found to be resistant to osmotic lysis when suspended in sorbitol at concentrations in the range 1.2M to 0.8M as judged by the decrease in absorbance which microscopic examination confirmed to have been caused by rupture of the sphaeroplasts. However further lowering of the molarity led rapidly to rupture of the entire population. In contrast, sphaeroplasts enriched with either campesterol, 7-dehydrocholesterol, 22-23 dihydrobrassicasterol or β-sitosterol were stable in sorbitol solutions with concentrations from 1.2M to 1.0M, but rapidly lost stability as the molarity of sorbitol was lowered further. The only difference in structure between the two groups of sterols is the presence

of a double bond at C-22 in ergosterol and stigmasterol; all of the remaining sterols have a saturated side chain at C-17. It would appear that the degree of unsaturation in ring B of the sterol does not influence the response of the sphaeroplasts to osmotic stress, since ergosterol has two double bonds (at C-5 and C-7) as does 7-dehydrocholesterol, whereas all of the other sterols have only one double bond at C-5. Also the size of the alkyl group at C-24 and its configuration (R or S) do not appear to have any effect, since campesterol, 22-23 dihydrobrassicasterol and β-sitosterol all have similar effects on plasma-membrane stability. While ergosterol and 22-23 dihydrobrassicasterol have an alkyl group at C-24 in the S configuration, campesterol, β-sitosterol and stigmasterol have a group in the R configuration.

Before considering the reasons for the two different types of response to osmotic lysis it should be emphasized that membranes, both artificial and natural, which are most stable are, in all probability, those in which the lipids present (phospholipids and sterols) exhibit the strongest interaction with each other (Demel et al., 1972b). Such interactions have been studied by measuring the lowering of the mean molecular area occupied by a phospholipid at an air-water interface following the addition of sterol to the monolayer. This is known as the 'condensing' effect and is studied using a Langmuir trough. It has been established that the degree of condensation depends on the nature of the fatty-acyl chains, with regard to chain

length and the degree of unsaturation (van Deenen et al., 1962; Demel et al., 1972a; Ghosh et al., 1973b), and also on the nature of the sterol molecule (Demel et al., 1972a; Ghosh and Tinoco, 1972). Other evidence has been obtained from e.s.r. data by Butler et al. (1970) who reported that sterols cause the fatty-acyl chains of sterol-free brain lipids to orientate themselves in a direction perpendicular to the lamellar plane, this being known as an 'ordering' effect. These latter workers also found that the mole % of sterol which gives a maximum ordering of the phospholipids varied with the nature of the sterol. The maximum ordering effect by cholesterol on sterol-free brain lipids occured at 25 mole % of the sterol, whereas ergosterol was more effective at 5 - 10 mole % (Butler et al., 1970). This range of mole ratios of sterol is the same as the proportion of sterol in anaerobically-grown Sacch. cerevisiae used during this study, and suggests that, if this proportion is the same in plasma membranes as in whole cells, Sacch. cerevisiae has a phospholipid : sterol ratio such that the membrane lipids interact most strongly.

The nature of the interaction between phospholipids and sterols has been studied by various workers. Shah and Schulman (1967) considered there was an absence of interaction between phospholipids and sterols, but that sterols fitted into cavities, the sizes of which were determined by the nature of the fatty-acyl chains. More recent work favours interaction between the lipids. From monolayer studies of phospholipid-sterol mixtures it was concluded

that the orientation of the hydroxyl group of the sterol was important as the degree of hydration and hydrogen bonding play an important part in lipid-lipid interactions (Demel et al., 1972a). The technique of n.m.r. has been used to examine the interactions of cholesterol and fatty-acyl chains of phospholipids. Darke et al. (1971) found that the sterol molecule and the first 8 - 10 methylene groups from the polar end of phosphatidylcholine are complexed by van der Waals forces. This interaction restricts the motion of these methylene groups, but those groups towards the methyl terminus of the fatty-acyl chain have much more freedom of motion. Similar evidence has also been found by using e.s.r. to study egg lecithin : cholesterol mixtures (Hubbell and McConnell, 1971) though these workers said that the first 8 carbon atoms could be considered to be a rigid rod in the presence of cholesterol.

I shall now attempt to explain the data obtained during the present study, by using these data already reported on phospholipid-sterol interactions.

When suspended in hypotonic solutions of sorbitol sphaeroplasts swell and the surface area occupied by the plasma membrane increases. Resistance to swelling will be conferred on those membranes which possess a sterol which interacts strongly with the fatty-acyl chains of the phospholipids. This in turn would render the sphaeroplasts stable to osmotic lysis, even in relatively low molarities of sorbitol. Results in this study suggest that

sphaeroplasts enriched with either ergosterol or stigmasterol exhibit the strongest interaction of any of the sterols used with the plasma membrane phospholipids, as these sphaeroplasts are more stable in hypotonic sorbitol solutions than sphaeroplasts enriched in other sterols. Measurement of sphaeroplast volumes support this view, since, in hypotonic solutions of sorbitol, surviving sphaeroplasts enriched with cholesterol swelled more rapidly than did those enriched with ergosterol.

The stronger interaction of ergosterol or stigmasterol must presumably be due principally to the presence of the double bond at C-22, particular attraction occurring between the double bond at C-22 of the sterol and C-9 of the phospholipid fatty-acyl chains, and this interaction stabilizes the membrane during osmotic stress. In contrast, sterols which have an alkyl group at C-24, but do not have a double bond at C-22 may disturb the packing of the fatty-acyl chains of the phospholipids, but not interact strongly enough to resist increase in area when present in hypotonic sorbitol. Cholesterol (and presumably 7-dehydrocholesterol although Butler et al., 1970, did not use this sterol) which lack a double bond at C-22 and an alkyl group at C-24 are presumably present at too low a concentration to cause appreciable interaction with the phospholipids, due to the small area occupied by the sterol side chain (Butler et al., 1970).

Preliminary work on monolayers of phospholipids and sterols

at an air-water interface were carried out during this study in order to attempt to explain the fragility of sphaeroplasts, and also to find if there could be interaction between a sterol and a phospholipid possessing a particular polar group. Interactions between sterols and phosphatidylcholines have often been studied by monolayer techniques (Demel et al., 1972a; Ghosh et al., 1972), as have interactions between cholesterol and phosphatidylethanolamine (Demel et al., 1967; Ghosh et al., 1973a). The three major phospholipids found in anaerobically grown Sacch. cerevisiae during this study were phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine. Of these only the first two were studied on monolayers. Phosphatidylcholine was selected as it is the most abundant phospholipid found in the yeast, and phosphatidylinositol was studied because there are no published data on its interactions with sterols, and also because there is a particularly high level in Sacch. cerevisiae (Longley et al., 1968; Getz et al., 1970; Hunter and Rose, 1972). Sterols used for the monolayer work were cholesterol and ergosterol, a representative from each group of sterol giving rise to osmotic sensitivity of sphaeroplasts.

The average area occupied on a monolayer by a phosphatidylcholine molecule was lowered by both cholesterol and ergosterol, although there were no appreciable differences between the condensing effects of the sterols even at molar ratios found during analyses of anaerobically-grown Sacch.

cerevisiae. This suggests that interaction with phosphatidylcholine is not dependent on the presence or absence of a double bond at C-22 of the sterol molecule. Although this contrasts with results obtained by Demel et al., (1972a) and Ghosh et al., (1972) it must be pointed out that these two groups used phosphatidylcholine which had fatty-acyl chains of a different composition from those examined in this study. It is possible that one or other of the sterols has particular attraction to particular unsaturated fatty-acyl chains on phospholipids as was reported by de Kruyff et al. (1973b).

However a study of the interactions between phosphatidylinositol and the sterols gave interesting results. It appeared that with phosphatidylinositol there was a 'condensing' effect by ergosterol at all molar ratios examined though not to the same extent as with phosphatidylcholine. But cholesterol, though it caused a lowering of the molecular area of phosphatidylinositol at high mole ratios of the sterol, the effect was not manifested at concentrations below 40 mole % cholesterol. Instead there was an apparent repulsion between the two lipids. This repulsion occurred at the phosphatidylinositol : sterol ratio found in anaerobically-grown Sacch. cerevisiae, which was 3 : 1. This effect could be due to repulsion of the hydroxyl groups on the inositol residue by the hydroxyl groups of the sterol, causing the inositol moiety of the phospholipid to lie below the plane of the monolayer, thus occupying a lower area per molecule. As

the proportion of cholesterol is lowered, the phosphatidylinositol molecules may move back into the plane of the monolayer thus increasing the area occupied by each lipid molecule. Repulsion by the hydroxyl groups could then force the lipid molecules apart to give an increase in average molecular area per lipid. On the other hand in the presence of ergosterol, which has a double bond at C-22, there might be, in addition to mutual repulsion by the hydroxyl groups of the lipids, an interaction between the side chain of the sterol and fatty-acyl residues of phosphatidylinositol particularly between the double bonds at C-22 of the sterol and C-9 of the fatty-acyl chains of the phospholipid. This latter effect may be stronger than that at the polar end of the lipid molecules, and result in a 'condensing' effect at all molar ratios. Caution must be applied when interpreting these monolayer data, as the phosphatidylinositol used in this study was from wheat and contained a high proportion of $C_{18:2}$ fatty-acyl residues, one hardly ever encountered in Sacch. cerevisiae.

It must be pointed out that these attempts to explain osmotic fragility are probably applicable only at low proportions of sterols. When present in higher mole ratios, compared to phospholipids, steric hindrance caused by the C-17 side chains with alkyl groups at C-24 could give rise to a lowered stability of the plasma membrane with subsequent loss of resistance to swelling whereas sterols such as cholesterol may confer stability on the membrane by lowering

the average area per lipid molecule. This is in agreement with results obtained by monolayer work (Demel et al., 1972a) and liposome permeability (Demel et al., 1972b). However it is possible that, in the yeast plasma membrane, there are clusters of sterol and phospholipid molecules which are in a 1 : 1 molar ratio, as has been previously reported by Darke et al. (1971) who used the technique of n.m.r. to examine codispersions of lecithin and cholesterol. If this does occur in the yeast plasma membrane, then the results reported in this thesis suggest that these clusters are composed of sterol and phosphatidylinositol, where there is a difference in interaction in the presence of different sterols, rather than sterol and phosphatidylcholine where there is no difference. This contrasts with the model proposed by Rothman and Engelman (1972) in which the molar ratios of sterols and phospholipids were not considered to be important, and steric hindrance by the sterol was thought to be responsible for membrane stability.

A particularly interesting feature of the data reported in this thesis is that it indicates a fundamental difference in the behaviour of membranes enriched in cholesterol, the typical mammalian sterol, compared with ergosterol, a typical fungal sterol. It is possible that these differences in behaviour of membranes are relevant to the distributions of these sterols in living organisms. It could be related to the temperature of growth of an organism. Saccharomyces cerevisiae grows optimally around

30°C whereas mammals maintain themselves around 37°C. At 30°C the fatty-acyl chains of phospholipids in yeast may be less fluid than at 37°C. However the bulkier side chain of ergosterol would tend to fluidize these lipids more readily than would cholesterol, but may cause steric hindrance at 37°C, giving rise to a less stable membrane at this temperature than would cholesterol.

The ability to stretch is important in the phenomenon of pinocytosis. The plasma membranes of plant protoplasts, as examined by electron microscopy, were distorted locally by latex spheres of 0.12μm diameter, suggesting that adhesive forces are involved with the initiation of invagination (Willison et al., 1971). Stretching would cause the density of the plasma membrane to be lower at the site of invagination, so that only this part of the membrane will stretch and surround the particle. The capacity for stretching and the resulting stability of the membrane could be determined by the structural characteristics of the membrane lipid molecules, particularly the sterols. Whether the ability of a membrane to affect pinocytosis is coupled with the presence in that membrane of a sterol with a saturated side chain will only be confirmed when plant and animal cell plasma membranes have been studied for sterol composition.

Another reason for differences between animal and fungal sterols may be due to a possible function of animal sterols

with insertion of glycolipids, a type not encountered in fungi, in the cell wall.

The data reported in this thesis demonstrate that cells and membranes of Sacch. cerevisiae can be altered in a specific manner, affecting the nature of sterols, and that this alteration may be exploited to examine the effect of sterol structure on physical properties of the yeast plasma membrane.

BIBLIOGRAPHY

Adams, B.G. and Parks, L.W. (1967) J. Cell. Physiol. 70, 161.

Adams, B.G. and Parks, L.W. (1968) J. Lipid Res. 9, 8.

Alterthum, F. and Rose, A.H. (1973) J. Gen. Microbiol. 77, 371.

Andreasen, A.A. and Stier, T.J.B. (1954) J. Cell. Comp. Physiol. 43, 271.

Babij, T., Moss, F.J. and Ralph, B.J. (1969) Biotechnol. Bioeng. 11, 593.

Baraud, J., Cassagne, C., Genevois, L. and Joneau, M. (1967) C. R. Acad. Sci. Ser. D 265, 83.

Baraud, J., Maurice, A. and Napias, C. (1970) Bull. Soc. Chim. Biol. 52, 421.

Barton, D.H.R., Harrison, D.M. and Widdowson, D.A. (1968) Chem. Commun. 17.

Bills, C.E., Massengale, O.N. and Puckett, P.S. (1930) J. Biol. Chem. 87, 259.

Bloomfield, D.K. and Bloch, K. (1960) J. Biol. Chem. 235, 337.

Boggs, J.M. and Hsia, J.C. (1972) Biochim. Biophys. Acta 290, 32.

Boulton, A.A. (1965) Exp. Cell Res. 37, 343.

Breivik, O.N., Owades, J.L. and Light, R.F. (1954) J. Org. Chem. 19, 1734.

Brown, C.M. and Johnson, B. (1970) J. Gen. Microbiol. 64, 279.

Brown, C.M. and Rose, A.H. (1969) J. Bacteriol. 99, 371.

Bulder, C.J.E.A. (1971) Antonie van Leeuwenhoek; J. Microbiol. Serol. 37, 353.

Butler, K.W., Smith, I.C.P. and Schneider, H. (1970) Biochim. Biophys. Acta 219, 514.

Cartledge, T.G. and Rose, A.H. (1973) Proceedings of the Third International Specialized Symposium on Yeasts (Suomalainen, H. and Waller, C., Eds.) p. 251, Oy, Helsinki.

Castelli, A., Barbaresi, G. and Bertoli, E.I. (1969) Ital. J. Biochem. 18, 91.

Chan, S.I., Feigensen, G.W. and Seiter, C.H.A. (1971) Nature (London) 231, 110.

Chang, S.B. and Matson, R.S. (1972) Biochem. Biophys. Res. Commun. 46, 1529.

Chapman, D., Byrne, P. and Shipley, G.G. (1966) Proc. Roy. Soc. Ser. A 290, 115.

Chapman, D., Williams, R.M.and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445.

Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756.

Clausen, M.K., Christiansen, K., Jenson, P.K. and Behnke, O. (1974) FEBS Lett. 43, 176.

Cobon, G.S. and Haslam, J.M. (1973) Biochem. Biophys. Res. Commun. 52, 320.

Corner, T.R. and Marquis, R.E. (1969) Biochim. Biophys. Acta 183, 544.

Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1971) FEBS Lett. 18, 326.

Davson, H. and Danielli, J.F. (1935) J. Cell. Comp. Physiol. 5, 495.

Dawson, P.S.S. and Craig, B.M. (1966) Can. J. Microbiol. 12, 775.

Dean, P.D.G., Ortiz De Montellano, P.R., Bloch, K. and Corey, E.J. (1967) J. Biol. Chem. 242, 3014.

de Duve, C., Berthet, J. and Beaufay, H. (1959) Progr. Biophys. Biophys. Chem. 9, 326.

de Gier, J., Mandersloot, J.G. and van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 150, 666.

Deierkauf, F.A. and Booij, H.L. (1968) Biochim. Biophys. Acta 150, 214.

de Kruyff, B., Demel, R.A. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 331.

de Kruyff, B., De Greef, W.J., van Eyk, R.V.W., Demel, R.A., and van Deenen, L.L.M. (1973a) Biochim. Biophys. Acta 298, 479.

de Kruyff, B., Demel, R.A., Slotboom, A.J., van Deenen, L.L.M. and Rosenthal, A.F. (1973b) Biochim. Biophys. Acta 307, 1.

de Kruyff, B., van Dijck, P.W.M., Goldbach, R.W., Demel, R.A. and van Deenen, L.L.M. (1973c) Biochim. Biophys. Acta 330, 269.

de Kruyff, B., van Dijck, P.W.M., Demel, R.A., Schuijff, A., Brants, F. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 1.

Demel, R.A., Kinsky, S.C., Kinsky, C.B. and van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 150, 655.

Demel, R.A., Bruckdorfer, K.R. and van Deenen, L.L.M. (1972a) Biochim. Biophys. Acta 255, 311.

Demel. R.A., Bruckdorfer, K.R. and van Deenen, L.L.M. (1972b) Biochim. Biophys. Acta 255, 321.

Demel, R.A., Geurts.van Kessel, W.S.M. and van Deenen, L.L.M. (1972c) Biochim. Biophys. Acta 266, 26.

Diamond, R.J. and Rose, A.H. (1970) J. Bacteriol. 102, 311.

Dittmer, J.C. and Lester, R.L. (1964) J. Lipid Res. 5, 126.

Dubé, J., Setterfield, G., Kiss, G. and Lusena, C.V. (1973) Can. J. Microbiol. 19, 285.

Dulaney, E.L., Stapley, E.O. and Simpf, K. (1954) Appl. Microbiol. 2, 371.

Dunphy, P.J., Kerr, J.D., Pennock, J.F., Whittle, K.J. and Feeney, J. (1967) Biochim. Biophys. Acta 136, 136.

Eddy, A.A. and Rudin, A.D. (1958) Proc. Roy. Soc. Ser. B 148, 419.

Eddy, A.A. and Williamson, D.H. (1957) Nature (London) 179, 1252.

Engelman, D.M. (1971) J. Mol. Biol. 58, 153.

Erwin, J.A. (1973) in Lipids and Biomembranes of Eukaryotic Micro-organisms (Erwin, J.A., Ed.), p. X1 Academic Press, London and New York.

Fieser, L.F. and Fieser, M. (1959) Steroids, Reinhold Publishing Corporation, New York.

Fryberg, M., Oehlschlager, A.C. and Unrau, A.M. (1973) J. Amer. Chem. Soc. 95, 5747.

Furukawa, Y. and Kimura, S. (1971) J. Vitaminol. 17, 219.

Gaines, G.L. (1966) Insoluble Monolayers at Liquid-Gas Interfaces, p. 30, J. Wiley and Sons, London and New York.

Gerard, E. (1895) J. Pharm. Chim. 1, 601.

Getz, G.S., Jakovcic, S., Heywood, J., Frank, J. and Rabinowitz, M. (1970) Biochim. Biophys. Acta 218, 441.

Ghosh, D. and Tinoco, J. (1972) Biochim. Biophys. Acta 266, 41.

Ghosh, D., Lyman, R.L. and Tinoco, J. (1973a) Lipids 8, 61.

Ghosh, D., Williams, M.A. and Tinoco, J. (1973b) Biochim. Biophys. Acta 291, 351.

Gollub, E.G., Trocha, P., Liu, P.K. and Sprinson, D.B. (1974) Biochem. Biophys. Res. Commun. 56, 471.

Gorter, E. and Grendel, F. (1925) J. Exp. Med. 41, 439.

Hanahan, D.J. and Olley, J.N. (1958) J. Biol. Chem. 231, 813.

Harrison, J.S. and Trevelyan, W.E. (1963) Nature (London) 200, 1189.

Harvey, A.J. (1968) in Methods in Cell Physiology (Prescott, D.M., Ed.), Vol. 3, p.1, Academic Press, London and New York.

Haskell, B.E. and Snell, E.E. (1965) Arch. Biochem. Biophys. 112, 494.

Hemming, F.W. (1970) Biochem. Soc. Symp. 29, 105.

Henry, S. (1973) in Lipids and Biomembranes of Eukaryotic Micro-organisms (Erwin, J.A. Ed.), p. 259. Academic Press, London and New York.

Hirschmann, H. (1960) J. Biol, Chem. 235, 2762.

Holden, M. and Tracey, M.V. (1950) Biochem. J. 47, 407.

Hosono, K. and Aida, K. (1974) J. Gen. Appl. Microbiol. 20, 47.

Hossack, J.A., Wheeler, G.E. and Rose, A.H. (1973) in Yeast, Mould and Plant Protoplasts (Villnueva, J.R., García-Acha, I., Gascón, S. and Urubu, F., Eds.), p. 211. Academic Press, London and New York.

Hsia, J.C., Long, R.A., Hruska, F.E. and Gesser, H.D. (1972) Biochim. Biophys. Acta 290, 22.

Huang, L., Jaquet, D.D. and Haug, A. (1974) Can. J. Biochem. 52, 483.

Hubbell, W.L. and McConnell, H.M. (1971) J. Amer. Chem. Soc. 93, 314.

Hunter, K. and Rose, A.H. (1971) in The Yeasts (Rose, A.H. and Harrison, J.S., Eds.), Vol.2, p. 211, Academic Press, London and New York.

Hunter, K. and Rose, A.H. (1972) Biochim. Biophys. Acta 260, 639.

Illingworth, R.F., Rose, A.H. and Beckett, A. (1973) J. Bacteriol. 113, 373.

Indge, K.J. (1968) J. Gen. Microbiol. 51, 425.

I.U.P.A.C. - I.U.B. Commission on Biochemical Nomenclature (1967), 127.

Jakovcic, S., Getz, G.S., Rabinowitz, M., Jakob, H. and Swift, H. (1971) J. Cell. Biol. 48, 490.

Johnson, B., Brown, C.M. and Minnikin, D.E. (1973) J. Gen. Microbiol. 75, x.

Jollow, D., Kellerman, G.M. and Linnane, A.W. (1968) J. Cell. Biol. 37, 221.

Jost, P., Libertini, L.J., Herbert, V.C. and Griffith, O.H. (1971) J. Mol. Biol. 59, 77.

Karst, F. and Lacroute, F. (1973) Biochem. Biophys. Res. Commun. 52, 741.

Karst, F. and Lacroute, F. (1974) Biochem. Biophys. Res. Commun. 59, 370.

Karunakaran, V. and Johnston, J.R. (1974) J. Gen. Microbiol. 81, 255.

Kates, M. and Baxter, R.M. (1962) Can. J. Biochem. Physiol. 40, 1213.

Kerridge, D. (1958) J. Gen. Microbiol. 19, 497.

Klyne, W. (1965) The Chemistry of the Steroids, Methuen

and Co. Ltd., London.

Kotyk, A. (1972) Biochem. J. 127, 51p.

Kováč, L., Sŭbík, J., Russ, G. and Kollár, K. (1967) Biochim. Biophys. Acta 144, 94.

Kubitischek, H.E. (1969) in Methods in Microbiology (Norris, J.R. and Ribbons, D.W., Eds.), Vol. 1, p. 593, Academic Press, London.

Kuksis, A. (1967) in Lipid Chromatographic Analysis (Marinetti, G.V., Ed.) Vol, 1, p. 239, Edward Arnold London.

Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333.

Lapper, R.D., Paterson, S.J. and Smith, I.C.P. (1972) Can. J. Biochem. 50, 969.

Leathes, J.B. (1925) Lancet 208, 853.

Lennarz, W.J. and Bloch, K. (1960) J. Biol. Chem. 235, p.c. 26.

Lester, R.L. and Steiner, M.R. (1968) J. Biol. Chem. 243, 4889.

Letters, R. (1966) Biochim. Biophys. Acta 116, 489.

Letters, R. (1968a) Bull. Soc. Chim. Biol. 50, 1385.

Letters, R. (1968b) in Aspects of Yeast Metabolism (Mills, A.K., Ed.) p. 306, Blackwells Scientific Publications, Oxford.

Levine, Y.K. and Wilkins, M.H.F. (1971) Nature (London)

230, 69.

Light, R.J., Lennarz, W.J. and Bloch, K. (1962) J. Biol. Chem. 237, 1793.

London, Y., Demel, R.A., Geurts van Kessel, W.S.M., Zahler, P. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 332, 69.

Longley, R.P., Rose, A.H. and Knights, B.A. (1968) Biochem. J. 108, 401.

Lynen, F. (1967) Biochem. J. 102, 381.

MacFarlane, M.G. (1964) Advan. Lipid Res. 2, 91.

Madyastha, P.B. and Parks, L.W. (1969) Biochim. Biophys. Acta 176, 858.

Maguigan, W.H. and Walker, E. (1940) Biochem. J. 34, 804.

Mangnall, D. and Getz, G.S. (1973) in Lipids and Biomembranes of Eukaryotic Micro-organisms (Erwin, J.A., Ed.), p. 145, Academic Press, London and New York.

Marchalonis, J.J., Cone, R.E. and Santer, V. (1971) Biochem. J. 124, 921.

Matile, Ph., Moor, H. and Mühlethaler, K. (1967) Arch. Mikrobiol. 58, 201.

Mendoza, C.G. and Villanueva, J.R. (1967) Biochim. Biophys. Acta 135, 189.

Meyer, F. and Bloch, K. (1963a) J. Biol. Chem. 238, 2654.

Meyer, F. and Bloch, K. (1963b) Biochim. Biophys. Acta 77, 671.

Moore, P.R. and Baumann, C.A. (1952) J. Biol. Chem. 195, 615.

Morpurgo, G., Serlupi-Crescenzi, G., Tecce, G., Valente, F. and Venettacci, D. (1964) Nature 201, 897.

Neudoerffer, T.S. and Lea, C.H. (1966) J. Chromatogr. 21, 138.

Noble, A.C. and Duitschaever, C.L. (1973) Lipids 8, 655.

Nurminen, T. and Suomalainen, H. (1970) Biochem. J. 118, 759.

Nurminen, T. and Suomalainen, H. (1971) Biochem. J. 125, 963.

Nurminen, T., Oura, E. and Suomalainen, H. (1970) Biochem. J. 116, 61.

Oldfield, E. and Chapman, D. (1971) Biochem. Biophys. Res. Commun. 43, 610.

Oldfield, E. and Chapman, D. (1972a) FEBS Lett. 21, 303.

Oldfield, E. and Chapman, D. (1972b) FEBS Lett. 23, 285.

Oldfield, E., Marsden, J. and Chapman, D. (1971) Chem. Phys. Lipids 7, 1.

Overton, E. (1899) Vierteljahresschr. Naturforsch. Ges. Zuerich 44, 88.

Palamarczyk, G. and Chojnacki, T. (1973) FEBS Lett. 34, 201.

Pecsok, R.L. (1959) in Principles and Practice of Gas Chromatography (Pecsok, R.L., Ed.) p. 135, John Wiley

and Sons, New York.

Phillips, D.R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284.

Ponsinett, G. and Ourisson, G. (1965) Memoirs Soc. Chim. 5, 3682.

Proudlock, J.W., Wheeldon, L.W., Jollow, D.J. and Linnane, A.W. (1968) Biochim. Biophys. Acta 152, 434.

Pugh, E.L. and Kates, M. (1973) Biochim. Biophys. Acta 316, 305.

Reindel, F., Weickmann, A., Picard, S., Luber, K. and Turula, P. (1940) Justus Liebigs Ann. Chem. 544, 116.

Resnick, M.A. and Mortimer, R.K. (1966) J. Bacteriol. 92, 597.

Robertson, J.D. (1959) Biochem. Soc. Symp. 16, 3.

Rothman, J.E. and Engelman, D.M. (1972) Nature 237, 42.

Rottem, S., Hubbell, W.L., Hayflick, L. and McConnell, H.M. (1970) Biochim. Biophys. Acta 219, 104.

Rottem, S., Pfendt, E.A. and Hayflick, L. (1971) J. Bacteriol. 105, 323.

Rottem, S., Yashouv, J., Ne'eman, Z. and Razin, S. (1973a) Biochim. Biophys. Acta 323, 495.

Rottem. S., Cirillo, V.P., De Kruyff, B., Shintzky, H. and Razin, S. (1973b) Biochim. Biophys. Acta 323, 509.

Schibeci, A., Rattray, J.B.M. and Kidby, D.K. (1973) Biochim. Biophys. Acta 311, 15.

Schreirer-Mucillo, S., Marsh, D., Dugas, H., Schneider, H. and Smith, I.C.P. (1973) Chem. Phys. Lipids 10, 11.

Schultz, J. and Lynen, F. (1971) Eur. J. Biochem. 21, 48.

Sentandreu, R. and Elorza, M.V. (1973) in Yeast, Mould and Plant Protoplasts (Villanueva, J.R., García-Acha, I., Gascón, S. and Urubu, F., Eds.) p. 187, Academic Press, London and New York.

Sentandreu, R. and Northcote, D.H. (1969) J. Gen. Microbiol. 55, 393.

Shafai, T. and Lewin, L.M. (1968) Biochim. Biophys. Acta 152, 787.

Shah, D.O. and Schulman, J.H. (1967) J. Lipid Res. 8, 215.

Shaw, N. and Baddiley, J. (1968) Nature (London) 217, 142.

Shaw, W.H.C. and Jefferies, J.P. (1953) Analyst 78, 509.

Shoppee, C.W. (1964) The Chemistry of the Steroids, Butterworths, London.

Singer, S.J. and Nicolson, G.L. (1972) Science, 175, 720.

Skidmore, W.D. and Enteman, C. (1962) J. Lipid Res. 3, 471.

Smedley-Maclean, T. and Thomas, E.M. (1920) Biochem. J. 14, 483.

Smith, S.W. and Lester, R.L. (1974) J. Biol. Chem. 249, 3395.

Stanacev, N.Z. and Kates, M. (1963) Can. J. Biochem. Physiol. 41, 1330.

Starr, P.R. and Parks, L.W. (1962a) J. Cell. Comp.

Physiol. 59, 107.

Starr, P.R. and Parks, L.W. (1962b) J. Bacteriol. 83, 1042.

Steim, J.M., Tourtellotte, M.E., Reinert, J.C., McElhaney, R.N. and Rader, R.L. (1969) Proc. Nat. Acad. Sci. U.S. 63, 104.

Steiner, M.R. and Lester, R.L. (1970) Biochemistry 9, 63.

Steiner, S. and Lester, R.L. (1972) Biochim. Biophys, Acta 260, 82.

Steiner, S., Smith, S.W., Waechter, C.J. and Lester, R.L. (1969) Proc. Nat. Acad. Sci. U.S. 64, 1042.

Sumper, M. and Träuble, H. (1973) FEBS Lett. 30, 29.

Suomalainen, H. and Nurminen, T. (1970) Chem. Phys. Lipids 4, 247.

Talamo, B., Chang, N. and Bloch, K. (1973) J. Biol. Chem. 248, 2738.

Tanner, W., Jung, P. and Behrens, N.H. (1971) FEBS Lett. 16, 245.

Tanret, C. (1889) Compt. Rend. 108, 98.

Tattrie, N.H. and Cyr. R. (1963) Biochim. Biophys. Acta 70, 693.

Trappe, W. (1938) Biochem. Z. 296, 180.

Trevelyan, W.E. (1968) J. Inst. Brew. 74, 365.

Työrinoja, K., Nurminen, T. and Suomalainen, H. (1974) Biochem. J. 141, 133.

Usden, V.R. and Burrell, R.C. (1952) Arch. Biochem.

Biophys. 36, 172.

van Deenen, L.L.M. (1972) Biochem. J. 128, 19p.

van Deenen, L.L.M., Houtsmuller, U.M.T., De Haas, G.H. and Mulder, E. (1962) J. Pharm. Pharmacol. 14, 429.

Wagner, H. and Zofcsik, W. (1966) Biochem. Z. 346, 343.

Watner, H., Horhammer, L. and Wolff, P. (1961) Biochem. Z. 334, 175.

Weete, J.D. (1973) Phytochemistry 12, 1843.

Welch, J.W. and Burlingame, A.L. (1973) J. Bacteriol. 115, 464.

Weinert, M., Kljaić, K. and Prošstenik, M. (1973) Chem. Phys. Lipids 11, 83.

Wheeler, G.E., Michell, R.H. and Rose, A.H. (1972) Biochem. J. 127, 64p.

Wieland, H. and Benend, W. (1942) Z. Phys. Chem. 274, 215.

Wieland, H. and Coutelle, G. (1941) Justus Liebigs Ann. Chem. 548, 270.

Wieland, H., Pasedach, H. and Ballauf, A. (1937) Justus Liebigs Ann. Chem. 529, 68.

Wilkie, D. (1970) Symp. Soc. Gen. Microbiol. 20, 381.

Willett, J.D., Sharpless, K.B., Lord, K.E., van Temelen, E.E. and Clayton, R.B. (1967) J. Biol. Chem. 242, 4182.

Williams, M.A., Taylor, D.W., Tinoco, J., Ojakian, M.A. and Keith, A.D. (1973) Biochem. Biophys. Res. Commun. 54, 1560.

Williams, R.M. and Chapman, D. (1970) in Progress in the

Chemistry of Fats and Other Lipids (Holman, R.T., Ed.) Vol. X1, p. 1, Pergamon Press, London.

Willison, J.H.M., Grout, B.W.W. and Cocking, E.C. (1971) J. Bioenergetics 2, 371.

Wisnieski, B.J. and Kiyomoto, R. (1972) J. Bacteriol. 109, 186.

Yuan, C. and Bloch, K. (1961) J. Biol. Chem. 236, 1277.

Environmentally Induced Changes in the Lipid Composition of Cells and Membranes of *Saccharomyces cerevisiae*

J. A. HOSSACK, G. E. WHEELER AND A. H. ROSE

Microbiology Laboratories, School of Biological Sciences, University of Bath, Bath, England

I. INTRODUCTION

During the first half of this Century, the biochemist was conspicuously successful in charting the major metabolic pathways that occur in living cells. With this knowledge available, it seemed inevitable that cell biologists would attempt to discover the intracellular location of individual metabolic processes. More recently, cell biologists have added a vectorial component to the overall picture provided by metabolic maps, and this has led to a renaissance in cell physiology. This development was accompanied by an interest in relationships between composition and function of cell membranes which form a permeability barrier between a cell and its environment and, by delimiting subcellular organelles, give rise to several discrete metabolic compartments within the cell.

To date, research has inevitably concentrated on biological membranes prepared from those tissues and organisms that are amenable to laboratory manipulation. The erythrocyte membrane and the Schwan cell membrane, which forms the myelin sheath, have been favoured by many workers. But, although these membranes can readily be obtained in bulk quantity, they are specialized membranes and so may not be ideal for studying basic membrane phenomena [1]. Other workers have turned to micro-organisms as convenient sources of biological membranes, largely because microbial membrane composition can be manipulated by altering the composition of the growth medium or other environmental conditions used for growing the organism. Attempts have been made to study the composition and function of several bacterial membranes, including (a) *Escherichia coli* (although because of the chemically complex cell wall in this bacterium it is not an ideal organism [2]); (b) strains of

Bacillus which can easily be converted into protoplasts; and (c) the mycoplasmas which do not form walls and so are attractive model organisms [3]. However, a major drawback to such studies on prokaryotic organisms is that the membrane composition is radically different from that of membranes derived from eukaryotic micro-organisms, and animal and plant cells.

An obvious solution to the problem of selecting an ideal model organism for composition-function studies on biological membranes is to choose a eukaryotic micro-organism, the membrane composition of which can be changed in a specific and predictable fashion. If the organisms selected can be studied and manipulated genetically so much the better. Strains of *Saccharomyces cerevisiae,* particularly those which are susceptible to conversion into sphaeroplasts, go a long way towards being ideal model organisms. This explains the considerable increase in research on yeast membranes in recent years.

The present paper shows how the lipid and membrane composition of *Sacch. cerevisiae* can be altered in a specific and predictable manner, providing a very useful method for studying the role of individual membrane components (e.g. phospholipids, sterols, fatty-acid residues) in membrane function.

II. Lipid Composition of *Saccharomyces cerevisiae*

For a long time, knowledge of the lipid composition of *Sacch. cerevisiae* was fragmentary compared with that available for other yeast-cell components. This situation simply reflected the fact that, for many years, studies on lipids lagged behind those on other classes of biological molecule, mainly because of the unattractive physical properties of lipids. Indeed, it is only since accurate and sensitive methods for analysis of lipids have become available that reliable data on the lipid composition of cells have been reported.

Several analyses of the lipid composition of *Sacch. cerevisiae* have been published [4]. From these analyses, it appears that the lipid composition of *Sacch. cerevisiae* resembles that of other eukaryotic organisms (Table 1). There are, however, important differences between the lipid composition of *Sacch. cerevisiae* and that of prokaryotic micro-organisms. Firstly, lipid extracts of *Sacch. cerevisiae* contain appreciable amounts of sterols (free and esterified) and of triacylglycerols, two classes of lipid that are virtually absent from prokaryotic micro-organisms [5]. Secondly, glycolipids are major components of Gram-positive bacteria and have also been detected in some Gram-negative bacteria [6, 7]. However, they are

TABLE I
Lipid content and composition of *Saccharomyces cerevisiae* NCYC 366

Lipid content (% dry weight cell)	Lipid composition (mg/100 mg dry weight cells)					
	Phospho-lipids	Triacylgly-cerols	Diacylgly-cerols	Free fatty acids	Free sterols	Sterol esters
12.5	3.76	1.22	0.09	0.38	0.23	1.32

Cells were grown batchwise at 30°C. Data from Hunter and Rose [15].

present in only very small amounts in *Sacch. cerevisiae* [8, 9]. Finally, this basic distinction between the lipid compositions of bacteria and *Sacch. cerevisiae* extends to the fatty-acid composition. Lipids from *Sacch. cerevisiae* contain predominantly residues of straight-chain saturated and unsaturated fatty acids, with chain lengths of 16 or 18 carbon atoms [10]. Bacterial lipids, on the other hand, contain fatty-acid residues with a greater variation in chain length, and also branched chain and cyclopropane fatty-acid residues [5].

Since the review by Hunter and Rose [4] was prepared, the main emphasis of research on yeast lipids has been on the quantitatively minor components. Reference has already been made to the recently published data on yeast glycolipids [8, 9]. In addition, Lester and his colleagues have made an intensive study of the inositol-containing phospholipids in *Sacch. cerevisiae*. At least seven of these have been detected [11]. The predominant representative is phosphatidyl-inositol, but there are also small amounts of diphosphatidylinositol and triphosphatidylinositol [12]. Four inositol-containing sphingolipids have been detected in *Sacch. cerevisiae* [11], the main component having the composition: ceramide-(P-inositol)$_2$-mannose [13].

III. Methods for Effecting Variations in the Lipid Composition of *Saccharomyces cerevisiae*

The great advantage of using micro-organisms in any physiological study is that their composition and metabolic activity can be manipulated, with some degree of precision, by varying the composition of the growth medium or other environmental conditions (such as growth temperature). There is a wealth of data available on ways in which environmental factors, such as medium composition, pH value, dissolved oxygen content and growth temperature, influence the lipid composition of bacteria [5] and yeasts [4].

When the aim is to use environmentally induced changes in the lipid composition of microbial membranes in studies on inter-relationships between composition and function it is highly desirable, and for definitive studies essential, that any change effected be stoicheiometric, i.e. that one lipid component be replaced by an equivalent amount of a closely related lipid. Although this ideal situation is difficult to achieve, only in this way can the role of individual components in membrane function be demonstrated.

The purpose of this contribution is to show how it is possible to achieve changes in the lipid composition of *Sacch. cerevisiae* approaching this ideal.

A. *Variations in Growth Temperature*

One of the best documented environmentally induced changes in the lipid composition of micro-organisms is the increase in proportion of unsaturated fatty-acid residues when the temperature at which a micro-organism is grown is lowered below the optimum for growth [14]. In this Laboratory we have examined the effects of variations in growth temperature as possible means of enriching the lipids in *Sacch. cerevisiae* NCYC 366 with unsaturated fatty-acid residues, as a prelude to a study of the role of lipid unsaturation in the functioning of the yeast plasma membrane. A detailed examination of the effect of growth temperature on the lipid composition of this yeast by Hunter and Rose [15] revealed that the changes induced by variations in growth temperature over the range 30°C to 15°C, either in batch-grown or continuously grown cells, are confined not only to variations in the degree of unsaturation of the fatty-acid residues in the lipids but also include changes in the amount of phospholipids synthesized. Indeed, with this strain of *Sacch. cerevisiae,* the proportion of unsaturation in cell lipids is much less affected, over this temperature range, than with many other yeasts including *Candida* strains [16]. Sphaeroplasts from cells of *Sacch. cerevisiae* NCYC 366 grown batchwise at 30°C or 15°C differ in their susceptibility to osmotic shock [17]. But the complex nature of the changes in lipid composition induced by lowering the growth temperature makes it impossible to state precisely the molecular basis of this difference in susceptibility.

B. *Provision of Exogenous Choline and Ethanolamine*

Saccharomyces cerevisiae is known to employ the methylation pathway for synthesis of phosphatidylcholine [4, 18]. The initial

step on this pathway is a reaction between CDP-diacylglycerol and serine to form phosphatidylserine, which is then decarboxylated to yield phosphatidylethanolamine. Phosphatidylcholine is then formed by N-methylation of phosphatidylethanolamine. A second pathway, the cytidine nucleotide pathway, operates in many although not all mammalian cells; its operation in eukaryotic micro-organisms has been very little studied.

However, there is evidence that the cytidine nucleotide pathway can be used in *Sacch. cerevisiae.* The pathway involves, initially, the formation of CDP-choline or CDP-ethanolamine from the free bases. These cytidine nucleotides then react with 1,2-diacylglycerols to produce, respectively, phosphatidylcholine and phosphatidylethanolamine. The nucleotides CDP-choline and CDP-ethanolamine have been detected in *Sacch. cerevisiae* by Kennedy and Weiss [19]. Subsequently, Lester and his colleagues [20, 21] reported incorporation of choline into phosphatidylcholine by *Sacch. cerevisiae,* and reported that the presence of choline in the growth medium represses synthesis of enzymes that catalyse synthesis of phosphatidylcholine by methylation of phosphatidylethanolamine.

Waechter and Lester [21] found that incorporation of choline in the growth medium leads to an increased synthesis of phosphatidylcholine by the yeast. This report has recently been confirmed in this Laboratory [22], and we have shown, in addition, that exogenous ethanolamine is incorporated into phosphatidylethanolamine by *Sacch. cerevisiae* NCYC 366 under conditions that also cause synthesis of additional amounts of phosphatidylethanolamine (Table II).

Growth of *Sacch. cerevisiae* in medium supplemented with choline or ethanolamine provides a useful means of obtaining cells that are enriched in phosphatidylcholine or phosphatidylethanolamine although, as yet, they have not been used to study the role of phosphatidylcholine and phosphatidylethanolamine in yeast membranes. A necessary prerequisite to these studies is the need to ascertain the distribution of the additional phospholipids in yeast membranes.

C. *Anaerobic Induction of Lipid Requirements*

Current work in our Laboratory employs a method for varying the lipid composition of *Sacch. cerevisiae* that approaches more closely the ideal situation described earlier. When strains of *Sacch. cerevisiae* are grown under strictly anaerobic conditions, they become auxotrophic for a sterol and an unsaturated fatty acid [23]. The

TABLE II

Effect of exogenous choline and ethanolamine on the phospholipid composition of *Saccharomyces cerevisiae* NCYC 366

Phospholipid	Content (μ mol phosphorus/g dry weight cells) in lipids from cells grown in			
	Unsupplemented medium	Medium supplemented with choline	Medium supplemented with ethanolamine	Medium supplemented with choline + ethanolamine
Phosphatidylcholine	10.7	39.2	15.7	24.4
Phosphatidylethanolamine	14.2	18.5	26.3	13.9
Phosphatidylinositol	13.9	11.1	16.2	14.4
Phosphatidylserine	1.0	3.4	1.9	2.9
Cardiolipin	2.9	2.2	1.5	0.8
Phosphatidic acid	2.7	1.8	5.9	1.0

Cells were grown batchwise at 30°C in basal medium [15] supplemented with choline (1 mM) and/or ethanolamine (10 mM). Data from Ratcliffe *et al.* [22].

specificities for these requirements have been examined, and both have been shown to be fairly broad. Thus, providing that the nutrient (sterol or fatty acid) supplied in the medium is incorporated unchanged into the yeast cell lipids, and this incorporation does not affect the regulation of synthesis of other lipids, the technique could prove valuable as a means of effecting specific changes in the composition of membranes in *Sacch. cerevisiae.*

Anaerobically Induced Fatty Acid Requirements. Bloch and his colleagues [24] found that anaerobically cultured *Sacch. cerevisiae* will not grow on stearic acid ($C_{18:0}$) but would when supplied with a C_{18} fatty acid that has a *cis* double bond between C-9 and C-10 or between C-10 and C-11, or a triple bond between C-9 and C-10. The 12-keto and 11, 12 cyclopropane derivatives supported growth less efficiently. Other acids, including the 10-methyl, 9, 10 epoxy, 9, 10 dihydroxy and 9, 10 *trans* olefinic derivatives, were inactive.

Professor Flavio Alterthum in our Laboratory, using these findings, has grown *Sacch. cerevisiae* NCYC 366 anaerobically in medium containing ergosterol, and oleic acid (18 : 1 *cis* 9 :10), linoleic acid (18 : 2 *cis* 9 : 10 12 : 13), or γ-linolenic acid (18 : 3 *cis* 6 : 7 9 : 10 12 : 13) [25]. Incorporation of any one of these acids in the medium does not affect the rate of growth of the yeast, but does change the duration of the lag phase of growth; this is greatest in medium supplemented with linolenic acid, and shortest in oleic acid-supplemented medium. The duration of the lag phase of growth was extended by about four hours as each acid was replaced by one with a greater degree of unsaturation. Cells grown in medium containing any one of these acids do not differ detectably in size or morphology. Moreover, the total lipid and total phospholipid contents of cells remain unaltered when the fatty-acid composition of the medium is varied. Analyses of the lipids from cells grown in media supplemented with oleic, linoleic, or linolenic acid show that fatty-acid supplement accounts for 65, 57 and 54%, respectively, of the total fatty-acid content (Table III).

Saccharomyces cerevisiae NCYC 366 possesses the considerable advantage of being readily convertible into sphaeroplasts by digesting the cell wall with snail gut juice or an exo-β (1→3) glucanase from Basidiomycete QM 806 [26]. Alterthum and Rose [25] went on to prepare sphaeroplasts from cells grown anaerobically in media supplemented with each of the different fatty acids. The susceptibility to sphaeroplast formation differs among cells grown in these three different media. Those grown in oleic acid-containing medium are converted into sphaeroplasts at a rate comparable with

TABLE III
Fatty-acid composition of lipids of *Saccharomyces cerevisiae* NCYC 366 grown anaerobically on different fatty acids

Fatty acid	Cells grown in medium containing Oleic acid	Linoleic acid	Linolenic acid
12 : 0	2.5	1.7	2.3
13 : 0	1.8	1.1	1.2
14 : 0	3.6	4.8	8.5
14 : 1	tr	1.5	—
16 : 0	18.5	24.2	22.8
16 : 1	4.0	2.1	2.4
18 : 0	2.3	2.7	5.7
18 : 1	65.1	5.2	1.3
18 : 2	1.6	56.7	1.9
18 : 3	—	—	53.9

Values indicate the percentage molar composition. Data from Alterthum and Rose [25].

that for cells grown aerobically. Cells grown in linoleic acid-containing medium are however more refractive and, in order to convert a population of these cells completely into sphaeroplasts in 45-60 min, it is necessary to double the concentration of glucanase protein in the digestion mixture. This finding indicates that the wall composition in these cells differs from that in oleic acid-grown cells. However, in spite of making further increases in the concentration of glucanase in the digestion mixture, it was not possible to convert cells grown in linolenic acid-containing medium into sphaeroplasts. Microscopically, sphaeroplasts can be seen to be formed from these cells, but they are extremely fragile and quickly lyse after extrusion. However, when spermine (10 mM) is included in the digestion mixture [27], it is possible to prepare stable sphaeroplasts of linolenic-grown cells. A plausible explanation of this finding is that spermine protects the extremely fluid plasma membrane of sphaeroplasts emerging from these cells.

This technique for obtaining sphaeroplasts with membranes that are enriched with oleic acid, linoleic or linolenic acid residues allowed Alterthum and Rose [25] to examine the effect of fatty-acid unsaturation on the susceptibility of the sphaeroplasts to osmotic lysis. Not surprisingly, perhaps, in view of the effect on the packing characteristics of phospholipids which contain fatty acyl chains with additional unsaturation, sphaeroplasts from cells grown in the presence of linolenic acid are more susceptible to osmotic lysis in

hypotonic solutions of sorbitol than those from cells grown in linoleic acid-containing medium, which in turn are more susceptible to lysis than sphaeroplasts from cells grown in oleic acid-supplemented medium.

The data of Alterthum and Rose [25] are the first to show how anaerobically-induced nutritional requirements of *Sacch. cerevisiae* can be exploited in studies on composition-function relationships in cell membranes. The technique offers numerous possibilities for further exploitation. Different series of fatty acids, including cyclopropane acids, could be examined in view of the broad specificity of the fatty-acid requirement. Other plasma-membrane functions, such as solute transport, might be examined. But probably the most attractive feature of the technique is that it permits a simultaneous examination of variations in the fatty-acid and the sterol composition of yeast membranes. This could be a considerable advantage in view of the suggestion [28] that sterols interact with unsaturated fatty acid acyl residues in membrane phospholipids.

Anaerobically Induced Sterol Requirements. The specificity of the sterol requirement of anaerobically grown *Sacch. cerevisiae* is easier to formulate than that for unsaturated fatty acids. It was examined by Linnane and his colleagues [29], who found, briefly, that the sterol must have a β-hydroxyl group at C-3, the molecule must be planar, and it must possess a long alkyl side chain at C-17.

One of us (J. A. Hossack, unpublished observations) exploited this broad specificity, and examined the growth and composition of *Sacch. cerevisiae* NCYC 366 in media supplemented with Tween 80 (polyoxyethylene sorbitan mono-oleate) to supply the unsaturated fatty acid, and ergosterol, cholesterol, or 7-dehydrocholesterol. These sterols were selected for a preliminary study, since ergosterol is a major yeast sterol, cholesterol is a sterol commonly found in mammalian tissues, and 7-dehydrocholesterol has an intermediate structure, with the steroid nucleus of ergosterol and a side chain identical with that of cholesterol.

There was no significant difference in the duration of the lag phase of growth or the rate of exponential growth of the yeast in media supplemented with the different sterols. Moreover, cells grown in each of the media do not differ either morphologically or in their contents of total lipids or total phospholipids. However, preliminary data show a difference in the amounts of free sterol and sterol ester in the lipids extracted from cells grown in each of the sterol-supplemented media. Ergosterol and cholesterol would seem to be incorporated largely unmodified and account for, respectively, 75

and 80% of the total free sterol in the cells. However, cells grown in media containing 7-dehydrocholesterol contain a high proportion of a sterol which has a retention time, on gas-liquid chromatography, similar to that of the tetraethenoid sterol which is a major component of *Sacch. cerevisiae* NCYC 366 [15]. A particularly interesting finding is that, although half of the sterols in cells grown in cholesterol-supplemented medium are esterified, cells grown on ergosterol contain very little sterol ester. It would seem that cells grown anaerobically on ergosterol could be used to study the kinetics of sterol-ester formation when they are incubated under aerobic conditions, in a way similar to that in which anaerobically grown yeast cells are used to investigate mitochondrial biogenesis.

D. *Use of Lipid-requiring Mutants*

An attractive alternative to using anaerobically induced lipid requirements of *Sacch. cerevisiae* is to isolate mutant strains of the yeast that are auxotrophic for a membrane lipid component or which have an altered lipid composition. Suitable medium supplementation will then permit controlled manipulation of the lipid composition of the yeast membranes. A number of mutants of this type have recently been described in the literature, and already some of these have been used to probe basic problems in membrane function. So far, just two classes of these mutants have been examined.

Unsaturated Fatty Acid-requiring Mutants. The first report of mutants of *Sacch. cerevisiae* with mandatory requirement for an unsaturated fatty acid for growth was by Resnick and Mortimer [30], who used ultraviolet irradiation of a wild-type haploid culture. Certain of these mutants have since been subject to extensive biochemical and genetical examination [31, 32]. In particular, a detailed examination has been made of the specificity of the requirement of these mutants for unsaturated fatty acids. All of those examined in detail are desaturase mutants, and must therefore be supplied with an unsaturated acid. The specificity of the requirement depends on the strain, but in general it can be met by any one of a wide range of acids, with chain lengths ranging from 14-20 carbon atoms, and with one, two or three double bonds in a variety of locations along the chain. Moreover, the double bond need not be *cis* [33]. The requirement for an unsaturated fatty acid by these mutants is therefore broader than the requirement by anaerobically grown *Sacch. cerevisiae*. The extent of enrichment of the cell lipids

by the fatty acid supplied varies between 50 and 75% of the total fatty acid [33].

A second series of unsaturated fatty acid-requiring mutants of *Sacch. cerevisiae* has been described, but as yet they have received only cursory examination. These are chain-elongation mutants [34, 35, 36] and have a nutritional requirement for a saturated fatty acid. They cannot grow when supplied with a highly purified unsaturated fatty acid.

So far, unsaturated fatty acid-requiring mutants of *Sacch. cerevisiae* have been employed mainly to study the dynamics of lipid alkyl chains in biological membranes using the technique of spin labelling [33, 37]. Reports have not yet appeared on formation of sphaeroplasts from these mutants, or on the effect, if any, which incorporating different unsaturated fatty acids into the cell lipids has on synthesis of the various classes of lipid. The use of these mutants in membrane studies has advantages, as well as disadvantages, compared with the use of anaerobically induced lipid requirements in *Sacch. cerevisiae.* Since they can be grown aerobically, they clearly are easier to use in the laboratory. And they can readily be subjected to genetical analysis. However, unsaturated fatty acid-requiring mutants cannot be used to study interactions in membranes between different sterols and unsaturated fatty acids. Nevertheless, there can be little doubt that the studies being made with these fatty acid-requiring mutants will help to elucidate interactions between individual components in biological membranes.

Sterol Mutants. Several groups of zymologists have attempted to isolate sterol-requiring mutants of *Sacch. cerevisiae,* but without success [38].

Recent research on polyene resistance in yeasts has revealed a way in which the sterol content and composition of *Sacch. cerevisiae* can be varied, and these findings could be useful to workers interested in composition-function relationships in yeast membranes. Polyene antibiotics complex with membrane-bound sterols, an interaction which severely disrupts the permeability of the plasma membrane [39]. Mutants of *Sacch. cerevisiae* that are resistant to one or more polyene antibiotics (etruscomycin, filipin, nystatin, pimaricin, rimocidin) have been isolated and, as expected, have an altered sterol composition compared with the parent strain [40, 41]. The possibility of exploiting these sterol mutants, particularly when coupled with a fatty-acid requirement, is attractive and could prove a valuable probe in membrane studies.

IV. Distribution of Lipids in Subcellular Structures of *Saccharomyces cerevisiae*

Establishing the nature and extent of environmentally induced changes in the lipid composition of *Sacch. cerevisiae* is only the first of a series of investigations, the aim of which is to explain relationships between composition and function in cellular membranes. If *Sacch. cerevisiae* is to be used as a model organism in membrane studies, it is essential first of all to establish the distribution of lipids in the various types of membrane (nuclear, mitochondrial, vacuolar, plasma), since it is a eukaryotic organism. When specific alterations in the lipid composition of *Sacch. cerevisiae* have been effected, it is then necessary to discover whether the lipid composition of all of the types of membrane in the cell are changed to the same extent, or whether the changes are confined to just one or two types of membrane.

Several preliminary studies have been reported on the separation of subcellular organelles in *Sacch. cerevisiae* [15, 42]. The most popular method is to prepare sphaeroplasts by digesting the cell wall, and to separate subcellular organelles from sphaeroplast lysates by differential, density-gradient or zonal centrifugation.

Having established methods for effecting specific alterations in the lipid composition of *Sacch. cerevisiae* NCYC 366, recent work in our Laboratory has been directed towards developing techniques for separating subcellular organelles from sphaeroplast lysates. As we are interested in both composition-function relationships in the plasma membrane, and the physiological roles of triacylglycerols and sterol esters, our work has concentrated on isolating plasma membranes and on establishing the subcellular location of triacylglycerols and sterol esters, using sucrose density gradients.

In order to establish rigorously the relative purity of subcellular fractions isolated by density-gradient centrifugation, it is essential to have available marker components or enzymes that are uniquely associated with a particular subcellular structure. These markers can then be used to monitor the distribution of individual subcellular structures on the gradient. Nuclear DNA and mitochondrial cytochromes are used to locate nuclei and mitochondria respectively. Marker compounds or enzymes for surface or plasma membranes are less well documented. In studies involving subcellular fractionation of animal cells, several enzyme activities have been used as markers for the surface membrane, including 5′-nucleotidase and $[Na^+ + K^+]$-stimulated ATPase [43, 44]. However, 5′-nucleotidase activity could not be detectable in subcellular fraction of *Sacch. cerevisiae* NCYC 366.

Because of the obvious need to isolate fractions rich in plasma membrane from *Sacch. cerevisiae* NCYC 366, one of us (G. E. Wheeler) investigated criteria for establishing the distribution of plasma membrane upon density-gradient centrifugation of lysed sphaeroplasts of *Sacch. cerevisiae* NCYC 366. Since there are no reports of enzyme activities specifically associated with the plasma membrane of yeast, initially an attempt was made to label chemically the plasma membrane of sphaeroplasts before lysis and fractionation. Marinetti and Gray [45] found that the fluorescent compound 4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulphonic acid (SITS) can bind specifically to the surface membrane of liver cells if the label is introduced before the cells are homogenized. Intact sphaeroplasts of *Sacch. cerevisiae* NCYC 366 were treated with SITS, excess reagent removed, and the sphaeroplasts lysed. The lysate was then submitted to density-gradient centrifugation, and the material at each interface of the discontinuous gradient collected. A qualitative determination of the concentration of SITS extracted from each fraction with potassium hydroxide indicated the distribution of plasma membrane on the gradient.

In our Laboratory, R. J. Diamond (unpublished observations) had previously obtained evidence for the presence of a $[Na^+ + K^+]$ -stimulated ATPase in crude plasma membranes from *Sacch. cerevisiae* NCYC 366; these membranes were obtained by differential centrifugation of lysed sphaeroplasts [17]. Subsequent experiments (G. E. Wheeler, unpublished observations) showed that while this activity can be found in crude plasma membrane fractions, and in fractions from density-gradient separations providing that the yeast cells have not been treated with a thiol reagent before or during sphaeroplast formation, nevertheless the activity could not consistently be demonstrated. Therefore, the activity is not a suitable marker enzyme for plasma membranes.

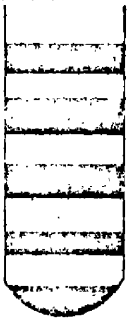
The enzyme phosphatidylinositol kinase has been shown to be located predominantly on the surface membranes of liver cells [46], and this activity was investigated as a possible plasma-membrane marker for *Sacch. cerevisiae* NCYC 366. The activity was detected in homogenates prepared either by lysing sphaeroplasts or by mechanical disruption of intact cells by shaking with glass beads. Phosphatidylinositol kinase activity was largely concentrated in material that collected at the interface between densities 1.13 and 1.16 on sucrose gradients [47]. When $[Na^+ + K^+]$ -stimulated ATPase activity could be detected, it too was largely concentrated in material with the same density as that containing phosphatidy-

linositol kinase with the highest specific activity. As surface membranes from mammalian cells have a density in this range [43], material derived from *Sacch. cerevisiae* NCYC 366 with densities in the range 1.13-1.16 was considered to be mainly plasma membrane.

A preliminary analysis of a fraction rich in plasma membrane from density gradients of lysed sphaeroplasts of *Sacch. cerevisiae* NCYC 366 (Table IV) shows that the lipid extracts contain predominantly phospholipid and sterol. Compared with whole-cell lipids, it is relatively deficient in sterol ester. These data indicate that the plasma membrane of *Sacch. cerevisiae* may have a lipid composition similar to that of animal cell-surface membranes [48].

TABLE IV

Lipid composition of fractions from a sucrose density-gradient of a sphaeroplast lysate of *Saccharomyces cerevisiae* NCYC 366

	Density	Phospholipids	Sterols	Sterol esters
	<1.08	27	29	52
	1.08-1.13	11	11	11
	1.13-1.16	12	14	1
	1.16-1.18	20	18	13
	>1.18	30	27	22

Values indicate percentage of total

Analyses of material from other density bands on sucrose gradients show that sterol esters are concentrated in organelles with a density below that of the plasma membrane-rich fraction (K. Hunter: Ph.D. Thesis; University of Bath). Analyses have been made of the low-density organelles (Table V) and these reveal that their composition resembles that of low-density lipid-containing vesicles from mammalian cells and plasma [49], that is they contain a large amount of lipid which is largely sterol ester and triacylglycerol. Low-density vesicles from *Sacch. cerevisiae* NCYC 366, like those from animal sources, are also precipitated by heparin in the presence of divalent cations.

Further work is required to establish the nature and physiological function of low-density lipid-containing vesicles from *Sacch. cerevisiae*. They could conceivably be associated with subcellular

TABLE V

Properties of low-density vesicles from *Saccharomyces cerevisiae* NCYC 366 compared with vesicles from mammalian systems

	High density lipoprotein	Low density lipoprotein	Very low density lipoprotein	Chylomicrons	Yeast vesicles
Lipid composition (%)					
Phospholipids	53	28	20	9	32
Triacylglycerols	14	14	42	89	26
Free sterols	33	10	8	0.8	3
Sterol esters		47	30	1.7	38
Density range	1.06-1.20	1.00-1.06	0.9-1.00	0.94	<1.08

Data on mammalian vesicles are from Margolis [49].

structures that have been identified as vacuoles [50]. Alternatively, they may be similar to the vesicles which may be involved with release of material from the plasma membrane during wall growth [51] or secretion of internally synthesized enzymes such as invertase [52]. It is attractive to assign such a role to these vesicles, at least provisionally, since an enzyme that catalyses hydrolysis of sterol esters has been located in the periplasmic space of *Sacch. cerevisiae* NCYC 366 [53], an activity which may be concerned with dissociation of enzymes and wall material from the vesicles.

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References

1. Rouser, G., Nelson, G. J., Fleischer, S. and Simon, G. (1968). *In:* Biological Membranes, p. 5 (Chapman, D. ed.). Academic Press, London and New York.
2. Silbert, D. F. and Vagelos, P. R. (1967). *Proc. natn. Acad. Sci. U.S.A.*, 58, 1579.
3. Razin, S. (1973). *Adv. microbial Physiol.* 10, 1.
4. Hunter, K. and Rose, A. H. (1971). *In:* The Yeasts, Vol. 2, p. 211 (Rose, A. H. and Harrison, J. S., eds). Academic Press, London and New York.
5. Goldfine, H. (1972). *Adv. microbial Physiol.*, 8, 1.
6. Shaw, N. (1970). *Bact. Rev.*, 34, 365.
7. Wilkinson, S. G. (1969). *Biochim. biophys, Acta*, 187, 492.
8. Baraud, J., Maurice, A. and Napias, C. (1970). *Bull. Soc. chim. Biol.*, 52, 421.
9. Brennan, P. J., Flynn, M. P. and Griffin, P. F. S. (1970). *FEBS Letters*, 8, 322.
10. Suomalainen, H. and Keränen, A. J. A. (1968). *Chem. Phys. Lipids*, 2, 296.
11. Steiner, S. and Lester, R. L. (1972). *J. Bact.*, 109, 81.
12. Lester, R. L. and Steiner, M. R. (1968). *J. biol. Chem.*, 243, 4889.
13. Steiner, S., Smith, S., Waechter, C. J. and Lester, R. L. (1969). *Proc. natn. Acad. Sci. U.S.A.*, 64, 1042.
14. Farrell, J. and Rose, A. H. (1967). *A. Rev. Microbiol.*, 21, 101.
15. Hunter, K. and Rose, A. H. (1972). *Biochim. biophys. Acta*, 260, 639.
16. Kates, M. and Baxter, R. M. (1962). *Can. J. Biochem. Physiol.*, 40, 1213.

17. Diamond, R. J. and Rose, A. H. (1970). *J. Bact.*, 102, 311.
18. Letters, R. (1966). *Biochim. biophys. Acta*, 116, 489.
19. Kennedy, E. P. and Weiss, S. B. (1956). *J. biol. Chem.*, 228, 195.
20. Waechter, C. J., Steiner, M. R. and Lester, R. L. (1969). *J. biol. Chem.*, 244, 3419.
21. Waechter, C. J. and Lester, R. L. (1971). *J. Bact.*, 105, 837.
22. Ratcliffe, S. J., Hossack, J. A., Wheeler, G. E. and Rose, A. H. *J. gen. Microbiol.*, 76, 445.
23. Andreasen, A. A. and Stier, T. J. B. (1954). *J. cell. comp. Physiol.*, 43, 271.
24. Light, R. J., Lennarz, W. J. and Bloch, K. (1962). *J. biol. Chem.*, 237, 1793.
25. Alterthum, F. and Rose, A. H. *J. gen. Microbiol.*, 77, 371.
26. Bauer, H., Bush, D. A. and Horisberger, M. (1972). *Experientia*, 28, 11.
27. Mager, J. (1959). *Biochim. biophys. Acta*, 36, 529.
28. Demel, R. A., Geuerts Van Kessel, W. S. M. and Van Deenen, L. L. M. (1972). *Biochim. biophys. Acta*, 266, 26.
29. Proudlock, J. W., Wheeldon, L. W., Jollow, D. J. and Linnane, A. W. (1968). *Biochim. biophys Acta*, 152, 434.
30. Resnick, M. A. and Mortimer, R. K. (1966). *J. Bact.*, 92, 597.
31. Keith, A. D., Resnick, M. R. and Hayley, A. B. (1969). *J. Bact.*, 98, 415.
32. Wisnieski, B. J., Keith, A. D. and Resnick, M. R. (1970). *J. Bact.*, 101, 160.
33. Wisnieski, B. J. and Kiyomoto, R. Y. (1972). *J. Bact.*, 109, 186.
34. Schweizer, E. and Bolling, H. (1970). *Proc. natn. Acad. Sci. U.S.A.*, 67, 660.
35. Schweizer, E., Kühn, L. and Castorph, H. (1971). *Z. Physiol. Chem.*, 352, 384.
36. Henry, S. A. and Keith, A. D. (1971). *Chem. Phys. Lipids*, 7, 284.
37. Eletr, S. and Keith, A. D. (1972). *Proc. natn. Acad. Sci. U.S.A.*, 69, 1353.
38. Adelberg, E. A., Roslansky, P. F., Myers, J. W. and Coughlin, C. A. (1955). *J. Bact.*, 69, 733.
39. Lampen, J. O. (1966). *Symp. Soc. gen. Microbiol.*, 16, 111.
40. Woods, R. A. (1971). *J. Bact.*, 108, 69.
41. Molzahn, S. W. and Woods, R. A. (1972). *J. gen. Microbiol.*, 72, 339.
42. Matile, Ph. (1970). *FEBS Symposium*, 20, 39.
43. Wheeler, G. E., Coleman, R. and Finean, J. B. (1972). *Biochim. biophys. Acta*, 255, 917.
44. Emmelot, P. and Bos, C. J. (1962). *Biochim. biophys. Acta*, 58, 374.
45. Marinetti, G. V. and Gray, G. N. (1967). *Biochim. biophys. Acta*, 135, 580.
46. Michell, R. H., Harwood, J. L., Coleman, R. and Hawthorne, J. N. (1967). *Biochim. biophys. Acta*, 144, 649.
47. Wheeler, G. E. and Rose, A. H. (1972). *Biochem J.*, 127, 64P.
48. Skipski, V., Barclay, M., Archibald, F. M., Terebuo-Kekish, O., Reichman, E. S. and Good, J. J. (1965). *Life Sci.*, 4, 1673.
49. Margolis, S. (1969). *In:* Structural and Functional Aspects of Lipoproteins In Living Systems, p. 369 (Tria, E. and Scanu, A. M., eds). Academic Press, London and New York.
50. Matile, Ph., and Wiemken, A. (1967). *Arch. Mikrobiol.*, 56, 148.
51. Sentandreu, R. and Northcote, D. H. (1969). *J. gen. Microbiol.*, 55, 393.
52. Beteta, P. and Gascon, S. (1971). *FEBS Letters*, 13, 297.
53. Wheeler, G. E. and Rose, A. H. (1973). *J. gen. Microbiol.*, 74, 189.

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Modifications to the Phospholipid Composition of *Saccharomyces cerevisiae* Induced by Exogenous Ethanolamine

By S. J. RATCLIFFE, J. A. HOSSACK, G. E. WHEELER AND A. H. ROSE
Microbiology Laboratories, School of Biological Sciences, Bath University, Bath

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INTRODUCTION

The lipid composition of micro-organisms is very responsive to changes in the chemical and physical properties of the environment. In many micro-organisms, altering one environmental factor frequently causes multiple changes in lipid composition. For example, although lowering the growth temperature of micro-organisms below the optimum usually leads to an increased synthesis of lipids with a high proportion of unsaturated fatty acid residues (Farrell & Rose, 1967*a*,*b*), in *Saccharomyces cerevisiae* this effect is accompanied by changes in the proportions of phospholipids (Hunter & Rose, 1972). Studies in this laboratory have exploited environmentally induced changes in the lipid composition of *Sacch. cerevisiae* (reviewed by Hunter & Rose, 1971) to investigate relationships between composition and function in yeast membranes. In these studies, it is desirable to be able to bring about specific changes in the lipid composition of organisms. Lester and his colleagues (Waechter, Steiner & Lester, 1969; Waechter & Lester, 1971) recently showed that the presence of choline (1 or 10 mM) in the growth medium leads to synthesis of an increased proportion of phosphatidylcholine by *Sacch. cerevisiae*. This was the first report showing how the content of one type of phospholipid in *Sacch. cerevisiae* can be altered by medium supplementation, and it provides a useful technique for varying the phosphatidylcholine content of yeast membranes. The present paper shows that provision of exogenous ethanolamine in the growth medium can similarly induce an increased synthesis of phosphatidylethanolamine by *S. cerevisiae*.

METHODS

The yeast used was *Saccharomyces cerevisiae* NCYC 366. Methods for maintaining the yeast, growing it in defined medium in batch culture, harvesting organisms, extracting lipids and separating and determining the contents of individual phospholipids were as described by Hunter & Rose (1972) except that, in some experiments, the phosphorus contents of phospholipids were directly determined by acid digestion of the materials without their removal from the thin-layer plates. Phospholipids on thin-layer plates were identified from R_F values of standard compounds. Ethanolamine was sterilized by membrane filtration and included in medium at 10 mM. Choline, which was included in medium at 1 mM, was sterilized by filtration or by autoclaving.

RESULTS

Addition of choline to the medium causes an increase of about 60 % in the synthesis of total phospholipid by *Saccharomyces cerevisiae* NCYC 366, as indicated by the phosphorus content of the lipids (Table 1). Most of this increase is attributable to an almost fourfold

Table 1. *Effect of exogenous choline and ethanolamine on the phospholipid composition of Saccharomyces cerevisiae* NCYC 366 *and incorporation of exogeneous ethanolamine into these phospholipids*

Choline was incorporated into the basal medium at 1 mM and ethanolamine at 10 mM. Organisms were harvested from cultures in the mid-exponential phase of growth (0·20 to 0·24 mg dry wt/ml), lipids extracted, and determinations made of the lipid phosphorus and the contents of individual phospholipids. Values quoted are the means of at least four separate determinations, where indicated ± standard errors of the mean. In experiments on incorporation of radioactive ethanolamine, organisms were grown in medium supplemented with [2-^{14}C]ethanolamine (1 μCi/mmol) with or without choline. Bands of silica gel containing labelled phospholipid were scraped off thin-layer plates, and transferred to scintillation vials containing 5 ml scintillation liquid (toluene: 2,5-diphenyloxazole; 1:0·003, v/w). Samples were counted in a Beckman liquid scintillation spectrometer (model 1650) for up to 100 min. Readings were corrected for average background count using blank areas of silica gel from thin-layer plates.

	Content (μmol phosphorus/g dry wt yeast) in lipids from organisms grown in:				Radioactivity (c.p.m./μmol phosphorus) in lipids from organisms grown in:	
Phospholipid	Unsupplemented medium	Medium supplemented with choline	Medium supplemented with ethanolamine	Medium supplemented with choline + ethanolamine	Medium supplemented with ethanolamine	Medium supplemented with choline + ethanolamine
Phosphatidylcholine	10·7 ± 0·3 (4)	39·2 ± 0·6 (4)	15·7 ± 0·5 (4)	24·4 ± 0·6 (4)	1339	12
Dimethylphosphatidylethanolamine	—	—	—	—	2336	0
Phosphatidylethanolamine	14·2 ± 0·8 (4)	18·5 ± 1·3 (4)	26·3 ± 0·4 (4)	13·9 ± 0·1 (4)	558	284
Phosphatidylinositol	13·9 ± 0·2 (4)	11·1 ± 1·3 (4)	16·2 ± 3·8 (4)	14·4 ± 0·4 (4)	98	32
Phosphatidylserine	1·0 ± 0·1 (4)	3·4 ± 0·3 (4)	1·9 ± 0·7 (4)	2·9 ± 0·3 (4)		
Cardiolipin	2·9 ± 0·5 (4)	2·2 ± 0·4 (4)	1·5 ± 0·2 (4)	0·8 ± 0·2 (4)	0	0
Phosphatidic acid	2·7 ± 0·4 (4)	1·8 ± 0·8 (4)	5·9 ± 2·3 (4)	1·0 ± 0·3 (4)	0	0
Lipid phosphorus	44·5 ± 1·1 (4)	71·3 ± 0·1 (4)	66·7 ± 2·2 (4)	55·8 ± 1·4 (4)		

rise in the content of phosphatidylcholine. The increases in total phospholipid and in the content of phosphatidylcholine are somewhat greater than those reported by Waechter & Lester (1971). Supplementing the medium with ethanolamine causes a smaller increase in the synthesis of total phospholipids (Table 1). In these organisms, the increase is mainly attributable to synthesis of additional phosphatidylethanolamine and to a lesser extent of phosphatidylcholine. Organisms grown in medium supplemented with both choline and ethanolamine also synthesize more total phospholipid, but to a smaller extent than those grown in medium supplemented with only ethanolamine (Table 1). In organisms grown in the doubly supplemented medium, the increase in phospholipid is caused mainly by an increase in the phosphatidylcholine content; synthesis of other phospholipids was hardly affected as compared with organisms grown in unsupplemented medium. Small amounts of other phospholipids, including dimethylphosphatidylethanolamine, were detected in lipids from organisms grown in the various media, but the amount of any one of these lipids never exceeded 1 % of the total phospholipid. Addition of choline to medium had no effect on the duration of the lag phase or on the rate of growth of the yeast. The lag phase of growth was however extended by 2 to 4 h when ethanolamine was added to the medium; the rate of exponential growth was not affected.

Label from [2-^{14}C]ethanolamine is incorporated into phosphatidylethanolamine by organisms grown in choline-free medium (Table 1). Essentially all of the label was shown

to be in the ethanolamine moiety of the phosphatidylethanolamine. Phospholipids were separated from neutral lipids in the extract by thin-layer chromatography (petroleum spirit + diethylether + acetic acid; 70:30:2, by vol) and hydrolysed by the procedure of Dittmer, Feminella & Hanahan 1958. Liberated ethanolamine was separated by descending paper chromatography using methylethyl ketone + methoxyethanol + 20 % (v/v) acetic acid (40:15:20, by vol), and the spot corresponding to ethanolamine cut out and extracted with 50 % (v/v) ethanol (Magee, Baker & Thompson, 1960). The radioactivity of the ethanolamine accounted for virtually all of the activity of the phosphatidylethanolamine. The recovery did not differ significantly from that obtained when a standard of [2-^{14}C]ethanolamine was submitted to the same procedure. However, in lipids from organisms grown in [2-^{14}C]ethanolamine-supplemented, choline-free medium, the specific activity of phosphatidylcholine is greater than that of phosphatidylethanolamine; moreover, the specific activity of dimethylphosphatidylethanolamine is greater than that of phosphatidylcholine (Table 1). In these experiments, it was not possible to resolve completely phosphatidylinositol and phosphatidylserine, and the radioactivity of these lipids is quoted as one value. When choline is added to [2-^{14}C]ethanolamine-supplemented medium, considerably less label from ethanolamine is incorporated into the lipids (Table 1), and almost all of that is in phosphatidylethanolamine the specific activity of which is nevertheless only half of that obtained with this phospholipid in extracts from organisms grown in choline-free medium.

DISCUSSION

Our data confirm the finding of Waechter *et al.* (1969) on the effect of exogenous choline on synthesis of phosphatidylcholine by *Saccharomyces cerevisiae*, and extend this effect to show that provision of exogenous ethanolamine causes a smaller but nevertheless reasonably specific increase in phosphatidylethanolamine synthesis. Organisms enriched in phosphatidylcholine or phosphatidylethanolamine will be useful in studies on the role of these phospholipids in yeast membranes, particularly since suitable methods have not so far been available for studying the role of these phospholipids in, for example, membrane transport. In addition, our data show that it is possible to label phosphatidylethanolamine in lipids of *Saccharomyces cerevisiae* with some degree of specificity, a technique which furnishes a valuable tool in studies on phospholipid metabolism in yeast.

Two different pathways lead to synthesis of phosphatidylcholine. One involves formation of phosphatidylserine from CDP-diacylglycerol and serine; phosphatidylserine is then decarboxylated to yield phosphatidylethanolamine which, in turn, is *N*-methylated to produce phosphatidylcholine. In a second pathway, CDP-choline reacts with a 1,2-diacylglycerol to produce phosphatidylcholine; phosphatidylethanolamine is synthesized in a similar reaction involving CDP-ethanolamine and a 1,2-diacylglycerol. The former or methylation pathway operates in a wide range of organisms including bacteria and fungi (Goldfine, 1972) and mammalian tissues (Lennarz, 1970). *Saccharomyces cerevisiae* uses the methylation pathway (Letters, 1966; Waechter *et al.* 1969; Hunter & Rose, 1971). However CDP-choline and CDP-ethanolamine were detected in *Sacch. cerevisiae* (Kennedy & Weiss, 1956; Tochikura, Kimura, Kawai & Gotan, 1972) which suggested that the second or cytidine nucleotide pathway may also be used by yeast. The data of Waechter *et al.* (1969) and Waechter & Lester (1971) on incorporation of choline into phosphatidylcholine provided the first direct evidence for operation of the cytidine nucleotide pathway in *Sacch. cerevisiae*. Our data on incorporation of label from [2-^{14}C]ethanolamine into phosphatidylethanolamine by *Sacch. cerevisiae* provide further direct evidence for operation of this pathway in this organism. Indirect evidence for synthesis of phosphatidylethanolamine from ethanolamine

came recently from Steiner & Lester (1972). They studied incorporation of *sn* [^{14}C]glycero-3[^{32}P]phosphate into phospholipids by a cell-free particulate fraction of *Sacch. cerevisiae*, and calculated that the cytidine nucleotide pathway may be responsible for about a quarter of the phosphatidylethanolamine synthesized by yeast. The possibility that ethanolamine is incorporated into phosphatidylethanolamine by reactions other than those of the cytidine nucleotide pathway appears unlikely. A Ca^{2+}-stimulated exchange of ethanolamine with phospholipids has been reported *in vitro* in animal (Dils & Hübscher, 1959) and plant tissue (Yang, Freer & Benson, 1967) although the role of the reaction *in vivo* is very uncertain (Hill & Lands, 1970). The reaction has not, however, been reported in micro-organisms. Even if such an exchange reaction were possible in yeast, the rapid rate of synthesis of phospholipids in exponentially growing cultures of yeast would probably preclude any major contribution to phospholipid synthesis being made by the reaction. Nevertheless, until data are available on the operation of the exchange reaction in yeast, the possibility that some ethanolamine is incorporated in this way cannot be dismissed. The possibility that ethanolamine is incorporated into phosphatidylserine following carboxylation of ethanolamine can be discounted since an ethanolamine carboxylase has not been reported.

The increased synthesis of phosphatidylethanolamine, but to a much more limited extent of phosphatidylcholine, in organisms grown in the presence of ethanolamine suggests that methylation of phosphatidylethanolamine is under tight metabolic control. However, the high specific activity of phosphatidylcholine and especially of dimethylphosphatidylethanolamine from organisms grown in choline-free ethanolamine-supplemented medium suggests a preferential methylation of phosphatidylethanolamine synthesized from exogenously provided ethanolamine. An alternative explanation by which free ethanolamine may be methylated to choline (Nemer & Elwyn, 1960) seems unlikely in view of the high specific activity of the dimethylphosphatidylethanolamine in these organisms. It is conceivable that methylation of phosphatidylethanolamine synthesized from phosphatidylserine, and of phosphatidylethanolamine synthesized from ethanolamine, occurs at different sites in yeast. The need for separate locations for these syntheses may be associated with the requirements of the organism for phospholipids with fatty-acid residues containing different degrees of unsaturation for, in animal tissues, it has been reported that phosphatidylcholine synthesized by the cytidine nucleotide pathway contains a greater proportion of saturated fatty-acid residues than when synthesized by the methylation pathway (Kanoh, 1969).

Our results on incorporation of labelled ethanolamine into phospholipids by organisms grown in the presence of choline support the suggestion of Waechter & Lester (1971) that the presence of free choline in the medium represses synthesis of enzymes that catalyse methylation of phosphatidylethanolamine to phosphatidylcholine. The contents of phosphatidylethanolamine and phosphatidylcholine in these organisms suggest that synthesis of phosphatidylethanolamine is controlled by choline since, even when methylation of phosphatidylethanolamine is repressed, the content of phosphatidylethanolamine in the organisms does not increase as compared with those grown in unsupplemented basal medium. The lower specific activity of phosphatidylethanolamine in these organisms also suggests that the presence of choline regulates synthesis of phosphatidylethanolamine from ethanolamine, possibly as a result of competition between choline and ethanolamine for a phosphokinase (Wittenberg & Kornberg, 1953).

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REFERENCES

DILS, R. R. & HÜBSCHER, G. (1959). The incorporation *in vitro* of [Me-^{14}C]choline into the phospholipids of rat-liver mitochondria. *Biochimica et biophysica acta* **32**, 293–294.

DITTMER, J. C., FEMINELLA, J. L. & HANNAHAN, D. J. (1958). A study of the quantitative estimation of ethanolamine and serine in phospholipids. *Journal of Biological Chemistry* **233**, 862–867.

FARRELL, J. & ROSE, A. H. (1967*a*). Temperature effects on micro-organisms. In *Thermobiology*, pp. 147–218. Edited by A. H. Rose. New York: Academic Press.

FARRELL, J. & ROSE, A. H. (1967*b*). Temperature effects on micro-organisms. *Annual Review of Microbiology* **21**, 101–120.

GOLDFINE, H. (1972). Comparative aspects of bacterial lipids. *Advances in Microbial Physiology* **8**, 1–58.

HILL, E. E. & LANDS, W. E. M. (1970). Phospholipid metabolism. In *Lipid Metabolism*, pp. 185–277. Edited by S. J. Wakil. New York: Academic Press.

HUNTER, K. & ROSE, A. H. (1971). Yeast lipids and membranes. In *The Yeasts*, vol. 2, pp. 211–270. Edited by A. H. Rose and J. S. Harrison. London: Academic Press.

HUNTER, K. & ROSE, A. H. (1972). Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. *Biochimica et biophysica acta* **260**, 639–653.

KANOH, H. (1969). Biosynthesis of molecular species of phosphatidyl choline and phosphatidyl ethanolamine from radioactive precursors. *Biochimica et biophysica acta* **176**, 756–763.

KENNEDY, E. P. & WEISS, S. B. (1956). The function of cytidine coenzymes in the biosynthesis of phospholipids. *Journal of Biological Chemistry* **222**, 193–214.

LENNARZ, W. J. (1970). Lipid metabolism. *Annual Review of Biochemistry* **39**, 359–388.

LETTERS, R. (1966). Phospholipids of yeast. II. Extraction, isolation and characterization of yeast phospholipids. *Biochimica et biophysica acta* **116**, 489–499.

MAGEE, W. L., BAKER, R. W. R. & THOMPSON, R. H. S. (1960). The identification and quantitative estimation of ethanolamine and serine in lipid hydrolysates. *Biochimica et biophysica acta* **40**, 118–123.

NEMER, M. J. & ELWYN, D. (1960). The conversion of serine to ethanolamine and its derivatives in the rat. *Journal of Biological Chemistry* **235**, 2070–2074.

STEINER, M. R. & LESTER, R. L. (1972). *In vitro* studies of phospholipid biosynthesis in *Saccharomyces cerevisiae*. *Biochimica et biophysica acta* **260**, 222–243.

TOCHIKURA, T., KIMURA, A., KAWAI, H. & GOTAN, T. (1972). Fermentative production of lipid-related substances. II. Effect of various factors on the production of cytidine diphosphate ethanolamine by yeasts. *Japanese Journal of Fermentation Technology* **50**, 178–181.

WAECHTER, C. J. & LESTER, R. L. (1971). Regulation of phosphatidylcholine biosynthesis in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **105**, 837–843.

WAECHTER, C. J., STEINER, M. R. & LESTER, R. L. (1969). Regulation of phosphatidylcholine biosynthesis by the methylation pathway in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **244**, 3419–3422.

WITTENBERG, J. & KORNBERG, G. A. (1953). Choline phosphokinase. *Journal of Biological Chemistry* **202**, 431–444.

YANG, S. F., FREER, S. & BENSON, A. A. (1967). Transphosphatidylation by phospholipase D. *Journal of Biological Chemistry* **242**, 477–484.